MOLECULAR SYSTEMATICS OF *Campylobacter* ISOLATED FROM THE HUMAN CLINICAL ENVIRONMENT

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LIST OF ABBREVIATIONS

bp	base pairs
CCDA	*Campylobacter* charcoal differential agar
cfu	colony forming units
dH_2O	distilled water
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
dNTP	2′-deoxy nucleotide triphosphates
kb	kilobase pairs
LPS	lipopolysaccharide
MDH	malate dehydrogenase NAD^+
ME	malate dehydrogenase $NADP^+$
min	minute(s)
MLEE	multilocus enzyme electrophoresis
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
x g	times gravity

ABSTRACT

Campylobacter jejuni is the major cause of campylobacteriosis in humans. This thesis recorded the distribution of *C. jejuni* and *C. coli* isolates in a hospital environment during 1997/98. 105 clinical isolates of *Campylobacter* were examined using *flaA* and *gmhA* PCR-RFLP analysis. Isolates were collected from 84 patients who presented at Christchurch Public Hospital or from the South Canterbury region. Males accounted for 63.1 % of the sample. The largest number of cases was reported in the age group 20-29 years. *flaA* specific primers were applied to all samples with 83.8 % generating a 1.7 kb amplicon. RFLP analysis using *Dde*I provided 17 different *flaA* profiles, with *flaA* 6 being the most common type identified in this study. 82.9 % of the sample generated a distinguishable profile. This technique provided a D value of 92 % (D = Simpson's index of diversity). *gmhA* primers were also applied to this sample, with 71.4 % generating either a 900 bp, 1.6 kb or multiple band profile. 62.9 % of the sample provided a distinguishable RFLP profile. *gmhA* 1 was the most commonly observed profile. This technique provided a D value of 74 %. When combining these two genetic markers, discrimination was increased. 59 % (n=62) of the isolates had provided both *flaA* and *gmhA* profiles. These isolates were distributed into 22 different classifications. MLEE analysis was applied to the largest *flaA* group in an attempt to further assess relationships. This analysis allowed *flaA* 6 to be subdivided into a further five groups, therefore increasing strain discrimination. PCR-RFLP procedures were highly reproducible, robust, discriminative and rapid to perform. However 17.1 % and 37.1 % were untypeable by *flaA* and *gmhA* respectively.

20 environmental (sheep) isolates from Massey University were also examined using *flaA* and *gmhA* PCR-RFLP analysis. Four *flaA* types and two *gmhA* types were observed amongst these isolates. The *flaA* and *gmhA* types had been previously observed in the clinical isolates, therefore suggesting that no strain was specific to a particular environment. However not all isolates were typeable with these two methods.

CHAPTER I

INTRODUCTION

1.1 DESCRIPTION OF THE GENUS.

1.1.1 BASIC MORPHOLOGY AND PHYSIOLOGY.

Members of the Genus *Campylobacter* are spiral or helical rods, 0.2 to 0.9 μm wide, 0.5 to 5 μm long, Gram-negative, non-spore-forming bacteria (Nachamkin 1992). Members of the Genus express polar, unsheathed flagellae and are actively motile using a corkscrew-like motion (Cover and Blaser 1989). The Generic name *Campylobacter* is derived from the Greek words *campylo*, meaning 'curved' and *bacter*, meaning 'rod' (Butzler and Skirrow 1979). Members of this Genus are important animal and human pathogens.

Originally classified within the Genus *Vibrio*, *Campylobacter* spp. differ in a number of key respects, particularly in DNA base composition and the ability to replicate under conditions of reduced oxygen tension (Corry *et al* 1995). The DNA of *Campylobacter* spp. is typified by being extremely A+T rich, with an average G+C content of 30 % (Nuijten *et al* 1990a, Chang and Taylor 1990, Taylor 1992b). Most *Campylobacter* spp. require a microaerobic atmosphere of 5-7 % oxygen, 10 % carbon dioxide and 80 % nitrogen (Corry *et al* 1995, Nachamkin 1992). The presence of hydrogen at >7 % concentration has been observed to improve primary isolation of *C. jejuni* from faeces (Corry *et al* 1995). While members of this Genus replicate across a wide range of temperatures, the human pathogens have an optimal growth temperature of 42°C. The so-called "thermophilic" *Campylobacter* are *C. jejuni, C. coli, C. upsaliensis* and *C. lari* (Ketley 1995). *Campylobacter jejuni* is believed to be responsible for 90 % of all human campylobacteriosis (Wallis 1994, Stonnet and Guesdon 1993).

1.1.2 TOLERANCE TO ENVIRONMENTAL STRESS.

Interaction of a variety of factors such as temperature, salt and pH can determine whether numbers of *Campylobacter* will increase, decrease or remain static. *C. jejuni*

survives in moist foods, such as milk and chicken, more readily at 42°C than at ambient temperatures. Thermal inactivation occurs at temperatures above 48°C, while freezing reduces the culturable bacterial numbers. *C. jejuni* is relatively sensitive to sodium chloride, desiccation and pH (optimal growth is at pH 6.5 – 7.5) (Christopher *et al* 1982, Hasell 1994). Under certain environmental conditions, such as exposure to atmospheric oxygen or nutrient limitation during stationary phase of growth, *Campylobacter* spp. undergo a transition in morphology to round or coccoid forms (Ketley 1995). The morphological transition is associated with the organism going from a viable and culturable form to a viable, non-culturable form. This transition is suggested as an adaptation to survival under adverse conditions (Rollins and Colwell 1986).

1.1.3 SIGNIFICANCE OF *Campylobacter* AS A HUMAN PATHOGEN.

Campylobacter spp. were first recognised in the early decades of this century as a cause of infectious abortion and infertility in sheep and cattle (Allos and Blaser 1995). In 1947 *C. fetus* was isolated from a pregnant woman who had had a septic abortion. This was the first reported case of pathogenicity in humans (Allos and Blaser 1995). In 1972 after the development of selective stool-culture techniques, *C. jejuni* was recognised as an important cause of acute diarrheal disease in humans (Allos and Blaser 1995). *C. jejuni* and *C. coli* appear to share many clinical and epidemiological characteristics (Blaser *et al* 1983) and campylobacteriosis caused by *C. jejuni* and *C. coli* is essentially indistinguishable. Therefore, interest in these organisms has been mainly for epidemiological purposes (Skirrow 1990). It may be possible that cases of campylobacteriosis due to *C. jejuni* may be over estimated due to difficulties in differentiating between *C. jejuni* and *C. coli* infection.

1.2 INCIDENCE OF CAMPYLOBACTERIOSIS.

In 1980 campylobacteriosis was made a notifiable disease in New Zealand and since then the incidence of infection has increased yearly (Faoagali 1984). For example, in December 1997 there were 1004 cases of campylobacteriosis notified, bringing the 1997 total to 8747 cases. This is compared to 817 notified cases for December 1996 and a total of 7628 cases in 1996. The rate of campylobacteriosis rose in 16 of the 24 health districts, with six regions being above the national average for 1997 (Public Health

Report 1998). Figure 1 shows the increase in campylobacteriosis notifications in New Zealand from 1980 to 1997.

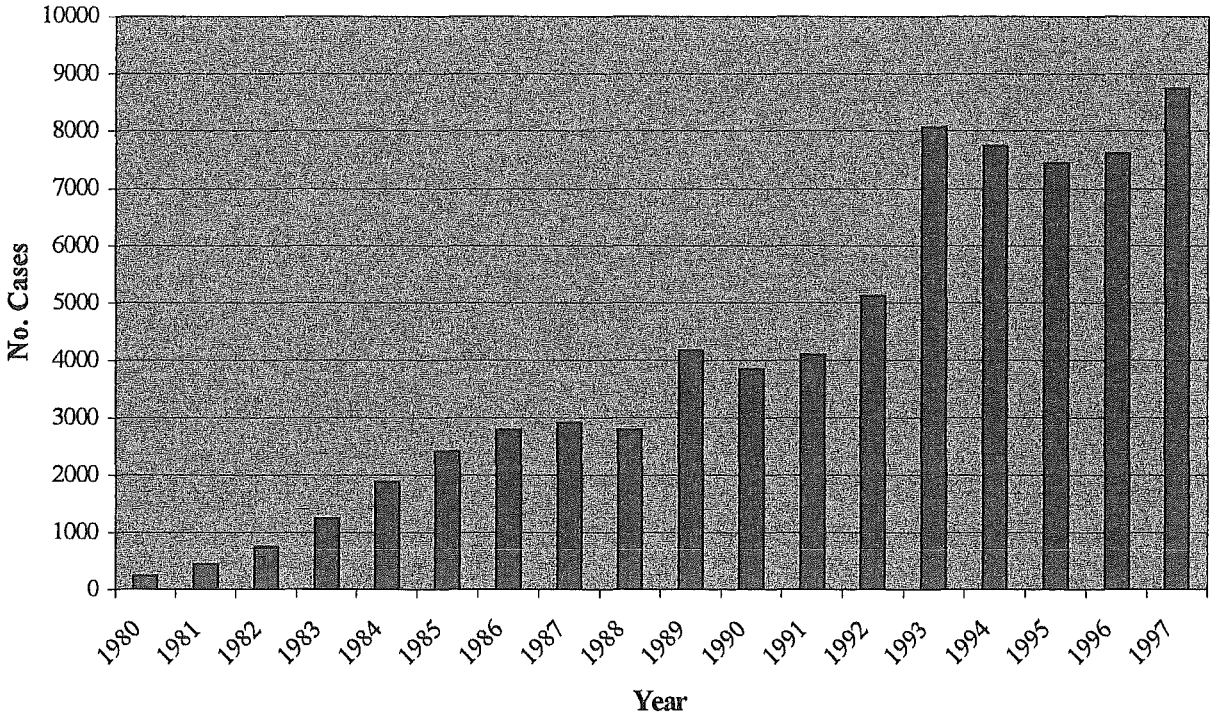


Figure 1: Increase in campylobacteriosis notifications in New Zealand from 1980 to 1997. (Adapted from the New Zealand Public Health Report and Communicable Disease New Zealand)

1.3 EPIDEMIOLOGY.

Males aged 1-4 and 20-29 years have the greatest incidence rate of campylobacteriosis. In fact, in all age categories males out-number females in rate of infection per 100,000 population (Allos and Blaser 1995, Butzler and Skirrow 1979, Blaser and Reller 1981). Brieseman (1994) observed that rural areas generally show a higher rate of infection than urban areas. Campylobacteriosis in the Canterbury region is primarily a spring/summer disease, peaking from November to March (Brieseman 1994). Figure 2 shows the campylobacteriosis notifications in Canterbury during 1996 and 1997.

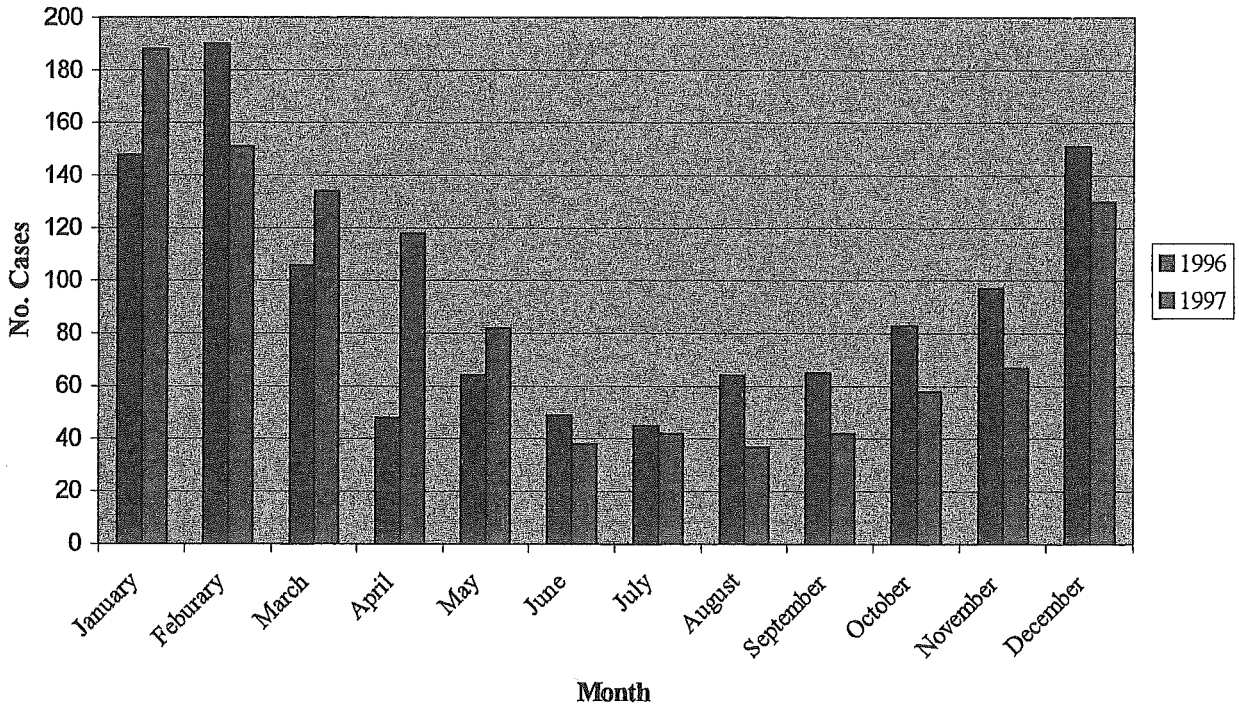


Figure 2: Campylobacteriosis notifications in Canterbury during 1996 and 1997.

There are a number of natural reservoirs for *Campylobacter* spp.. Avian species, especially wild birds, are the major animal reservoirs of *C. jejuni* (Altekruse *et al* 1994, Blaser and Reller 1981, Faoagali 1984, Rosef *et al* 1983). *C. jejuni* has been isolated from the faeces of 30 to 100 percent of chickens, turkeys, water fowl and other wild birds. *Campylobacter* spp. have also been isolated from non-human primates, coyotes, dogs, cats and hamsters (Altekruse *et al* 1994, Blaser and Reller 1981). Contact with infected animals can result in human infection. *C. jejuni* and other *Campylobacter* species are often present in stools of healthy dogs and those with diarrhea. Altekruse *et al* (1994) showed that dogs and cats with no signs of diarrhea were still positive for *Campylobacter* spp.. For instance in one clinic, 23 % of the dogs examined were asymptomatic carriers; while 21 % of the dogs examined were diseased.

Skirrow (1990) outlined three processes by which people become infected from animal flesh. These include:

1) handling of raw product,
2) consumption of raw or undercooked products,
3) cross-contamination of 'innocent' foods.

These scenarios are commonly observed with chicken consumption, or in the case of (3), consumption of salads or bread that have been cross-contaminated from raw meats. Chicken is frequently contaminated with *Campylobacters*, more so than red meats, and the level of contamination is often high. Pearson *et al* (1993) observed that in England and Wales, chicken was the second most common cause of *Campylobacter* infection. Most cases of campylobacteriosis from meat appears to be sporadic.

Raw milk was the first documented source of human campylobacteriosis (Altekruse *et al* 1994, Blaser *et al* 1983). *Campylobacter* spp. commonly present in raw milk are thought to result from faecal contamination at time of milking. Occasionally cows excrete organisms into their milk from *Campylobacter*-caused mastitis (Skirrow 1990, Altekruse *et al* 1994, Humphrey 1995). Robinson (1981) indicated that as little as 500 organisms of *C. jejuni* suspended in milk will result in infection. Doyle and Roman (1982) carried out studies on raw grade A milk and reaffirmed the need for pasteurisation to limit *C. jejuni*.

Water has also been linked to a number of outbreaks of campylobacteriosis. Waterborne outbreaks have been reported in the USA, Sweden, UK, NZ and elsewhere, and are generally associated with unchlorinated or under-chlorinated water (Brieseman 1987, Stehr-Green *et al* 1991, Blaser *et al* 1983). Brieseman (1987) examined an outbreak of *Campylobacter* enteritis in Ashburton, New Zealand, in 1986. It was found that the onset of the disease related to discolouration and lack of chlorination of the water supply. Heavy rain occurred a few weeks prior to the outbreak. It was postulated that heavy rainfalls washed *Campylobacter* from the surrounding beef and sheep farms into the river, affecting the town water supply. A delay in chlorination of the municipal water supply after the rain may have allowed a portion of untreated water to enter the system (Brieseman 1987). Similar observations have been made previously regarding suspected water outbreaks (Blaser *et al* 1983, Stehr-Green *et al* 1991). However,

C. jejuni has rarely been cultured from a suspected water supply, thereby complicating the story.

1.4 PATHOLOGY OF *Campylobacter* DISEASE.

1.4.1 CLINICAL ILLNESS.

The incubation period for campylobacteriosis ranges from one to seven days (Cover and Blaser 1989, Ketley 1995, Sahay *et al* 1995). The disease is characterised by symptoms such as fever, headache, muscular pain, nausea, vomiting and diarrhea (Humphrey 1995, Butzler and Skirrow 1979, Blaser *et al* 1983). Many patients have at least one day with eight or more bowel movements (Blaser *et al* 1983). Diarrhoeic stools often contain fresh blood, muco-pus and an inflammatory exudate with leucocytes (Ketley 1995). The faeces of patients who do not have chemotherapy usually remain positive for the organism for about two to five weeks after an attack of *Campylobacter* enteritis. In mild cases patients may excrete the organism for only a few days of infection (Butzler and Skirrow 1979).

Infective dose studies have been carried out to determine the number of organisms required for infection. Black *et al* (1988) found that 10^8 cells of *C. jejuni* resulted in a productive infection in 100 % of human volunteers in this trial (n=111), whereas as little as 500-800 bacteria suspended in milk or sodium bicarbonate could cause illness in >50 % of the sample. Black *et al* (1988) found the rate of infection (i.e., culture positive stools) increased with dose, but the development of illness did not show a clear dose response relationship. Infection with *Campylobacter* does not always produce symptoms: asymptomatic excretors are commonly found associated with enteritis patients (Butzler and Skirrow 1979). Individual susceptibility to the organism and the relative virulence of the infectious strain are both important factors in the pathogenesis of *Campylobacter* enteritis.

1.4.2 PREVENTION AND TREATMENT OF CAMPYLOBACTERIOSIS.

Enteric infection with *C. jejuni* is generally self-limiting, however relapses can occur in 20 % of untreated cases (Lacey 1993, Blaser and Reller 1981, Reina *et al* 1994). Amongst those who do seek medical attention 30 to 100 percent recover without

specific antimicrobial treatment (Skirrow 1977, Karmali and Fleming 1979). However, anecdotal reports indicate rapid resolution of symptoms and fewer relapses after antimicrobial therapy (Skirrow 1977, Blaser *et al* 1979). Control and prevention measures for *Campylobacter* infections are based on reduction of the reservoir in domestic animals or interruption of transmission to humans (Blaser *et al* 1983). Basic sanitary precautions are also required to prevent spread through animal contact.

As stated earlier, *C. jejuni* is found in the environment and intestine of many food-producing animals. Appropriate heating of foods and pasteurisation of milk are important measures useful in preventing infection (Altekruse *et al* 1994, Blaser *et al* 1983). Possible human to human transmission is also important to interrupt. Stools from infected infants and other incontinent persons represent the major human reservoir for transmission. Sanitary disposal of excreta and contaminated linens, and hand washing after contact are important to avoid infection (Blaser *et al* 1983, Lacey 1993). Patients suffering diarrhea should not follow usual employment if it involves catering or health care (Lacey 1993).

1.4.3 EXTRAINTESTINAL *Campylobacter* DISEASES.

Once established, a *Campylobacter* infection can manifest in several different ways. The vast majority of *C. jejuni* infection is gastrointestinal in nature, but extraintestinal infections, including meningitis, urinary tract infection, reactive arthritis and Guillain-Barré syndrome (GBS), have been reported (Walker *et al* 1986). GBS has become the most common cause of acute generalised paralysis, with an annual incidence of 0.75 to 2 cases per 100,000 population (Ropper 1992, Mishu *et al* 1993, Hughes 1991, Yuki *et al* 1995). GBS is an inflammatory, demyelinating disease, of peripheral nerves characterised by various degrees of weakness, sensory abnormalities and autonomic dysfunction (Hughes 1991, Ropper 1992). Although GBS has become the most common form of neuromuscular paralysis, its causes and pathogenesis are not clear (Rees *et al* 1995). In about two-thirds of patients, the neuropathic symptoms follow an often mild infection, such as an undiagnosed respiratory or gastrointestinal illness. Prior infection with *C. jejuni* is frequently associated with the onset of GBS (Rees *et al* 1995, Lacey 1993, Allos and Blaser 1995, Ho *et al* 1995, Yuki *et al* 1995, Mishu *et al* 1993, Aspinall *et al* 1994b). It has been postulated that onset of paralysis usually occurs

1-3 weeks after the initial bacterial infection (Ropper 1992). There appears to be no relationship between severity of gastrointestinal symptoms and development of GBS. Rees *et al* (1995) observed that men were more likely to develop GBS after a *C. jejuni* infection than women. This may suggest a sex-linked predisposition, or reflect the greater number of males infected with *C. jejuni* than females. In the United States and Japan 30 % - 80 % of *C. jejuni* isolates from patients with GBS belong to Penner serotype HS:19 (Allos and Blaser 1995, Kuroki *et al* 1991, Fujimoto *et al* 1992). The frequency of HS:19 strains amongst patients with gastroenteritis is often reported to be under 3 %, but may be as low as 1.2 % (M^{c}Myne *et al* 1982)

Examinations carried out by Aspinall and colleagues on the structure of lipopolysaccharide (LPS) extracted from several *C. jejuni* strains found that the terminal regions of core oligosaccharides (OSs) of several serotypes of *C. jejuni* resembled the structure of human gangliosides. Yuki *et al* (1995) speculated that infection by *C. jejuni* strains expressing ganglioside-like epitopes induces the enhanced production of anti-ganglioside antibodies in patients with certain immunologic backgrounds. This leads to loss of tolerance and the development of GBS. The commonly associated serotypes are HS:19 and HS:4, although other serotypes have been reported, these include HS:1, HS:2 and HS:10. Nachamkin *et al* (1996) observed, using polymerase chain reaction-restriction fragment length polymorphisms within the *flaA* gene, that 80 % of the HS:19 isolates were predominantly typed as *flaA* 21. However, other *flaA* types were evident, these included *flaA* 4 and *flaA* 70.

Miller-Fisher syndrome (MFS) has also been linked to a prior *C. jejuni* infection. MFS is a neuropathy associated with ataxia, areflexia and ophthalmoplegia (Salloway *et al* 1996). MFS is thought to occur when demyelination effects the cranial nerves.

1.4.4 PATHOGENESIS.

A reasonable understanding of the general clinical, microbiological and epidemiological aspects of *Campylobacter* spp. infection has been achieved. However, the mechanisms by which *Campylobacter* spp. induce disease are not well understood (Ketley 1995, Ruiz-Palacios 1992). The bacterial virulence factors, or determinants required to cause disease, are likely to be multifactorial in nature. *Campylobacter,* being a predominantly

foodborne pathogen, must be able to resist and survive physiological stresses (e.g., environmental factors) present in food and water. This ensures successful transmission to a susceptible host and ultimately infection. The ability of *Campylobacter* spp. to enter a viable but non-culturable state may be of significance to this survival (Ketley 1995). Mechanisms have been postulated by which *C. jejuni* induces illness. These include:

1) adherence, production of enterotoxins, multiplication, colonisation and resistance of host defences,
2) invasion and proliferation within the intestinal epithelium, resulting in cell damage and host inflammatory response (Ruiz-Palacios 1992, Ketley 1995, Cover and Blaser 1989).

1.5 TYPING METHODS.

1.5.1 INTRODUCTION.

The development of a suitable typing system to discriminate between isolates within a species and between Genera is essential for epidemiological studies. Typing methods can provide information regarding relationships between isolates and determine the extent and possible sources of disease outbreaks. Since the recognition of *Campylobacter* spp. as major causes of human gastrointestinal disease, different typing schemes have been established for use in epidemiological studies (Kaijser and Mégraud 1992). Systems based on phenotypic and genotypic traits, having varying degrees of simplicity and discrimination, have been developed. Genotypic methods appear to be most sensitive, as these systems have the advantage of measuring stable chromosomal differences. These results are more consistent than biochemical and phenotypic tests which are variable under different culture conditions, and can be applied to all species because they possess DNA (Kaijser and Mégraud 1992).

To be an effective epidemiological typing system several requirements must be met. These include:

1) typeability and/or robustness – the ability to give an unambiguous positive or negative result for each isolate,
2) reproducibility – the ability to provide an identical result when re-testing an isolate,
3) discriminatory power – the ability to differentiate between epidemiologically unrelated strains.

Additionally factors such as availability, cost, rapidity of the testing procedure and technical requirements (eg., equipment, personnel) also influence the typing system used.

The next section will present an overview of typing techniques that have been applied to *Campylobacter*, and commentary on the relative usefulness and potential problems associated with each system.

1.5.2 PHENOTYPIC TYPING METHODS.

1.5.2.1 Resistotyping.

Antimicrobial susceptibility testing (resistotyping) is a simple method to perform and interpret, measuring an isolate's resistance to various antimicrobial agents (Patton and Wachsmuth 1992). The usefulness of this test is dependent upon the organism's resistance to a variety of antimicrobial agents. Resistotyping has been found to correlate well with results from other schemes (Maslow and Mulligan 1996). Ribeiro *et al* (1996) examined *Campylobacter* resistance to a number of agents; tetracycline hydrochloride, metronidazole, 2,3,5-tetrazolium chloride, 5-fluorouracil, nalidixic acid and sodium arsenate. The level of reproducibility in this study was very good, with a test variation of <2 %. This research identified 35 resistotypes from 328 isolates, with the largest group consisting of 22 % of the isolates. Other investigators (Patton and Wachsmuth 1992) have found *C. jejuni* to be susceptible to most antimicrobial agents examined, therefore limiting the usefulness of this technique as a means for epidemiological typing. Resistotyping can be time consuming for *C. jejuni* when testing by agar MIC (minimal inhibitory concentration). However, *Campylobacter*

isolates received by the Center for Disease Control (CDC) are resistotyped to classify them into possible related groups.

1.5.2.2 Auxotyping.

Auxotyping has been used for typing of *Campylobacter* strains (Dickgiesser and Czylwik 1985, Tenover *et al* 1985, Tenover and Patton 1987). This technique exploits the different nutritional requirements necessary for growth of bacteria to distinguish between strains. In 1985 chemically defined media for auxotyping *Campylobacter* strains were developed (Dickgiesser and Czylwik 1985, Tenover *et al* 1985). This technique requires no special equipment, large numbers of isolates can be tested simultaneously and it is an easily reproducible method. *Campylobacter* auxotypes are consistent between laboratories and the system can be applied to other organisms with modification to accommodate diverse nutrient requirements (Sandstrom and Ruden 1990, Tebbutt 1984). Tenover and Patton (1987) identified 12 auxotrophic patterns from approximately 500 US isolates. However, in this study fewer than half of the *C. jejuni* strains and none of the *C. coli* strains, exhibited auxotrophic requirements. Therefore auxotyping has limited usefulness in epidemiological studies.

1.5.2.3 Biotyping.

Biotyping of *C. jejuni* and *C. coli* strains has been widely reported. This technique is relatively simple to use (Patton and Wachsmuth 1992). Biotyping is based on the presence or absence of a biochemical function, e.g., fermentation of glucose. Each test is assigned a numerical value if a positive result is produced or a zero for no result. The sum of these tests is referred to as a biotype. This technique is similar to that of auxotyping. This procedure is mainly used in clinical laboratories to identify an organism to Generic or species level. The Preston biotyping scheme uses 12 tests, including 10 resistotyping tests that determine resistance to antibiotics, dyes and chemicals. 55 biotypes of *C. jejuni* have been identified (Patton and Wachsmuth 1992). However Lior's biotyping scheme, which uses hippurate hydrolysis, rapid H_2S production and DNase activity, is the most widely used for distinguishing epidemiologically related strains, distinguishing 1,407 *C. jejuni* isolates into four biotypes (Lior 1984). Reproducibility of certain tests is poor and erratic, however this

may depend on the age of the culture medium, the incubation time or inoculum size of cultures, especially for the rapid H_2S test (Skirrow and Benjamin 1980).

1.5.2.4 Serotyping.

Serotyping detects antigenic determinants that are expressed on the surface of a microorganism using specific (polyclonal but absorbed) antisera. Two principle schemes have been developed for *C. jejuni*, these include:

1) Heat Stable (HS) antigens – developed by Penner and Hennessey (1980). Lipopolysaccharide (LPS) is the primary HS antigen used for differentiation of isolates in this scheme. This scheme identifies 60 serotypes (42 *C. jejuni* and 18 *C. coli*) (Aspinall *et al* 1993a, Moran 1991). Antisera are specific for each species, and species can be serotyped separately.
2) Heat Labile (HL) antigens – developed by Lior *et al* (1982), using outer membrane proteins as a marker, (e.g., flagella). This scheme identifies 108 serotypes (8 among *C. lari* strains) (Patton and Wachsmuth 1992).

Serotyping techniques are well developed and have been applied globally to identify *Campylobacter* strains (Patton and Wachsmuth 1992). However, serotyping of *C. jejuni* is problematic as both HS and HL schemes require considerable time and labour investment to produce antisera (Owen *et al* 1995). Also a significant number of strains are nontypeable by either method (Patton and Wachsmuth 1992). More importantly, Owen *et al* (1994) studied the discrimination of serotyping in relation to other techniques available, such as biotyping and PCR-RFLP. They found that the discriminatory power of serotyping alone does not meet the requirements of a modern typing scheme. There was molecular evidence that strains within each serogroup were genetically diverse. When Penner serotyping was used in conjunction with *flaA* typing (see below) discrimination of *C. jejuni* isolates increased from 20 serotypes to 28 unique clusters (Owen *et al* 1994). Penner serotyping and PCR fingerprinting are two completely different typing methods; Penner serotyping detects phenotypic and PCR fingerprinting detects genotypic differences (Aarts *et al* 1995) therefore combining these two methods provided an increased level of isolate discrimination.

1.5.3 GENETIC TYPING SCHEMES.

Molecular genotyping methods, based on chromosomal DNA analysis, are generally a more reliable method of characterising isolates as they are not dependent on expressed phenotypic features (Hernandez *et al* 1995). Several methods which exploit precise and stable strain markers of *Campylobacter* spp. have been developed. These include ribosomal DNA (rDNA) gene profiles obtained from Southern blot hybridisation and polymerase chain reaction (PCR) generated fingerprints using random primer sequences (RAPD-PCR) (Owen and Hernandez 1993). RAPD fingerprinting provides a discriminatory and rapid means of comparison of *Campylobacter* isolates (Hernandez *et al* 1995, Armstrong 1997). Other methods include pulsed field gel electrophoresis (PFGE) and PCR-restriction fragment length polymorphism (RFLP) based on various genes, (e.g., *flaA*).

1.5.3.1 Polymerase Chain Reaction (PCR).

The advent of PCR has greatly accelerated the progress of studies on the genomic structure of various organisms (Hayashi 1994). PCR is recognised as a powerful tool in the detection of genetic alterations. Amplification of a specific segment of genetic material allows direct analysis of the relevant region of DNA without the background of the entire complex genome (Daniell 1994). PCR has several advantages over other typing methods, these include rapidity of results; low input material, requiring only a few cells of the microorganism and non-complex protocols (Swaminathan and Barrett 1995). DNA can be prepared from fresh or frozen specimens using traditional extraction techniques. The simplest method of DNA preparation is to boil intact cells (Saiki *et al* 1986). This process simultaneously denatures and 'purifies' the DNA to allow PCR amplification. However, if too many cells (>50,000) are boiled, inhibition of the PCR can occur (Shibata 1994). Beyond simple detection of a pathogen, PCR technology can potentially provide genetic information relating to the virulence, chemosensitivity and epidemiological spread of a pathogen (Martin 1994). PCR has increasingly been used in the identification of many organisms. Viral infections due to the human immunodeficiency virus (HIV), human T-lymphocytotropic virus (HTLV) and hepatitis viruses are just a few that the PCR technique has been applied to in order to characterise the organisms involved. Parasitic and fungal infections can also be detected by PCR (Martin 1994). PCR offers a highly sensitive and specific alternative

to selective enrichment for the detection and identification of bacterial pathogens (Hill *et al* 1990, Lampel *et al* 1990, Bej *et al* 1991). Noteworthy infectious bacterial agents examined include *Mycobacterium* and *Borrelia burgdorferi*, as well as *Campylobacter*.

1.5.3.2 RFLP Typing Using the Flagellin Genes *flaA* and *flaB*.

The flagella of *C. jejuni* is now recognised as an important virulence factor required to colonise the intestine of a host (Taylor 1992b, Newell *et al* 1985, Morooka *et al* 1985, Black *et al* 1988, Aguero-Rosenfeld *et al* 1990, Pavlovskis *et al* 1991). The flagellin genes have been cloned and extensively characterised (Taylor 1992b, Ketley 1995, Nuijten *et al* 1990b). Two separate genes encoding flagellin, *flaA* and *flaB*, are present in the genome of *C. jejuni* and *C. coli* (Nuijten *et al* 1990b, Fischer and Nachamkin 1991, Guerry *et al* 1990). *flaA* and *flaB* are equally sized (approximately 1.7 kb), and are located adjacent to each other in a tandem (head to tail) orientation (Taylor 1992a). The *flaA* and *flaB* genes are transcribed independently and are regulated by different promoters (*flaA* uses σ^{28} whereas *flaB* uses a σ^{54} promoter) (Nuijten *et al* 1990b). *flaA* and *flaB* genes appear to be present in most of the *C. jejuni* and *C. coli* strains examined by DNA hybridisation analysis (Thornton *et al* 1990).

The flagellin gene *flaA* in *Campylobacter* spp. appears to have significant sequence heterogeneity and the gene has been used as a molecular epidemiological marker (Meinersmann *et al* 1997, Nachamkin *et al* 1993, Fischer and Nachamkin 1991, Thornton *et al* 1990). Most heterogeneity is found in the 5′, 3′ regions and a small central region of the gene (Harrington *et al* 1997, Ketley 1995, Sahay *et al* 1995, Waegel and Nachamkin 1996, Fischer and Nachamkin 1991). Nachamkin *et al* (1993) developed a PCR method to amplify the *flaA* gene of *C. jejuni* and used RFLP analysis of this gene to determine whether such a system would provide an alternative to serotyping. This PCR-based RFLP assay is sensitive, fast and reliable, and requires only minute amounts of DNA (<50 ng) (Nishimura *et al* 1996).

Although *fla* gene-based assays have been applied successfully to analysis of faecal material, they do not offer a primary distinction between *C. jejuni* and *C. coli* (Linton *et al* 1997). Comi *et al* (1996) observed that the *flaA* primers are closely related to *C. coli* and *C. jejuni* but could not detect *C. laridis* and other organisms. It was concluded from

this research that *flaA* typing could not discriminate between *C. jejuni* and *C. coli*. Hippurate hydrolysis or a second molecular technique must be used to discriminate between these species. The study carried out by Linton *et al* (1997) showed that PCR-based detection, identification to the species level and typing of *Campylobacter* directly from faecal samples, was possible for clinical laboratories. Meinersmann *et al* (1997) carried out multiple sequence analysis of *flaA* from *C. jejuni*; this indicated as much as 30 % difference in the gene from one isolate to another existed. This supports Nachamkin's observations that *flaA* typing could serve as an epidemiological marker. However PCR-RFLP assays are difficult to standardise and inter-laboratory comparisons of the results are especially difficult (Meinersmann *et al* 1997).

1.5.3.3 Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR).

RAPD-PCR fingerprinting of bacterial isolates was developed in 1990 (Welsh and M^{c}Clelland 1990, Williams *et al* 1990). RAPD-PCR techniques use genomic DNA as a template and an oligonucleotide primer of arbitrary single-stranded DNA sequence (Williams *et al* 1990). The ability of RAPD-PCR to discriminate between isolates is dependent upon the selection of a suitable random primer (Lam *et al* 1995). Hernandez *et al* (1995) observed that the RAPD-PCR method was a rapid means of comparing *Campylobacter* isolates and was discriminative in an intra-laboratory setting. However the DNA fragments generated are generally strain-specific; it is essential to know the Genus and species of the original organism. Usually the number of DNA fragments generated is dependent upon the ability of the primer sequence to detect a complimentary sequence in the target DNA. The distance of these sequences must be close enough to allow amplification, usually less than two kb apart. The generated profile may also depend upon the size of the target genome (Williams *et al* 1990).

There are several advantages of RAPD-PCR profiling. Swaminathan and Barrett (1995) observed that it is not necessary to have nucleotide sequence data available when designing primers to use. The same primers can be used for multiple applications. RAPD-PCR primers target the entire genome, resulting in a more complex and complete pattern than generated by PCR-RFLP procedures, therefore increasing the likelihood of demonstrating differences between strains. The PCR product does not

need to be purified, as restriction digesting of the PCR product is not performed. Berg *et al* (1994) found the RAPD method to be very sensitive, efficient and reliable.

The greatest disadvantage of this technique is the lack of reproducibility of results. A minor change in PCR conditions may result in a different RAPD profile (Micheli *et al* 1994, Mennier and Grimont 1993). Alterations in primer concentration, DNA template concentration and quality, *Taq* polymerase concentration and source, $MgCl_2$, the number of thermal cycles and even the model of thermal cycler used contribute to altering the reproducibility of this technique.

1.5.3.4 Ribotyping.

Ribotyping is a molecular technique based on hybridisation of a rRNA-DNA probe to restriction digested bacterial chromosomal DNA, measuring chromosomal differences. The technique involves generation of a labeled oligonucleotide probe, usually via PCR amplification of part of the 16S or 23S rRNA-DNA. The chromosomal DNA is digested with a restriction endonuclease and the digested DNA is transferred to a nitrocellulose or nylon membrane and probed with a radioactively labeled probe (Patton *et al* 1991). Ribotyping can generate data relating directly to gene copy number, as well as serving as an epidemiological marker (Stanley *et al* 1993, Stanley *et al* 1994). Ribotyping has proved to be a sensitive marker allowing for a high level of discrimination amongst isolates at the species level, thus distinguishing *C. jejuni* from *C. coli*. This technique along with other genotypic procedures has the potential to provide more consistent, reproducible results and is applicable to other Genera and species. However, discrimination is dependent on the enzyme and probe chosen for hybridisation (Mourean *et al* 1989, Owen *et al* 1990, Patton *et al* 1991). Ribotyping is a complex procedure which requires specialised reagents and equipment, and skilled staff (Patton *et al* 1991). For routine use this technique would need to be more rapid, have fewer complex steps and use a non-radioactive label. This is possible with the PCR DIG probe synthesis kit.

1.5.3.5 Pulsed-Field Gel Electrophoresis.

PFGE involves the digestion of chromosomal DNA into large (several hundred kilobases) fragments using rare cutting restriction endonucleases (RE). This technique

has become increasingly popular as an epidemiological tool (Tenover *et al* 1995), allowing differentiation between species. PFGE has been applied to *C. jejuni* for chromosome mapping studies and epidemiological classification of isolates (Chang and Taylor 1990, Kim *et al* 1992). However the sensitivity of PFGE depends on the choice of restriction enzyme (Patton and Wachsmuth 1992). Taylor (1992b) found the restriction enzymes *Sal*I and *Sma*I generated a manageable number of well-separated DNA fragments within the *C. jejuni* and *C. coli* chromosomes. Owen *et al* (1995) examined the discriminative ability of PFGE and found complete typeability of all *C. jejuni* strains was achievable. However, an inherent DNase activity present in some strains, which lead to premature DNA degradation, had to be overcome. This technique also requires expensive equipment, reagents and skilled staff. Significant time must also be spent on DNA preparation, electrophoresis and analysis.

1.5.3.6 Multilocus Enzyme Electrophoresis (MLEE).

MLEE has been used for many years in eucaryotic population genetics to estimate genetic diversity. More recently it has been used to estimate the genetic diversity and structure in natural populations of bacteria (Selander *et al* 1986). Selander's research established a basic population genetic framework to analyse variation in species of bacteria. This technique measures inheritable chromosomal characteristics by measuring the charge of enzymes, which translate to differences in enzyme migration. These results can be linked back to a specific change in the gene of an organism. MLEE has the ability to provide consistent and reproducible results. MLEE has proved useful as a marker for epidemiological studies, being based on the electrophoretic migration distances of enzymes present in most bacteria (Patton and Wachsmuth 1992). Different structural genes are represented by enzymes of cellular metabolism (Aeschbacher and Piffaretti 1989). Aeschbacher and Piffaretti (1989) observed that no electrophoretic type was shared between *C. jejuni* and *C. coli*, therefore indicating that this technique can distinguish between *Campylobacter* spp..

Data provided by MLEE allows identification of strains for epidemic purposes as well as acting as a measurement of genetic relatedness among strains within a species (Selander *et al* 1986, Aeschbacher and Piffaretti 1989, Patton *et al* 1991). Research carried out by Aeschbacher and Piffaretti (1989) found 50 and 14 electrophoretic types

among 104 *C. jejuni* and 21 *C. coli* strains respectively, demonstrating a high degree of genetic diversity within these two species. Unfortunately, MLEE is complex and is dependent on the number of enzymes analysed and the use of appropriate electrophoretic parameters (Patton and Wachsmuth 1992). In comparison to other genetic methods MLEE is relatively time consuming, for example PCR-RFLP analysis can be completed in 2 to 3 days, compared with 3 or more weeks required for MLEE (Patton and Wachsmuth 1992, Patton *et al* 1991). The technique may be best used to study population genetics of a large group of strains or where background data already exists (Patton and Wachsmuth 1992), for example, it could be used to further classify clinical isolates where a presumptive identification of *Campylobacter* has been made.

1.6 LIPOPOLYSACCHARIDE.

Lipopolysaccharide (LPS) is the major component (3-8 % dry weight of the cell) of the outer membrane of Gram-negative bacteria (Hancock *et al* 1994, Nikaido and Vaara 1985). It is responsible for many of the protective properties of the outer membrane. LPS has been shown to contribute to several aspects of the infection process including eliciting cytotoxic effects, immunity to serum complement and protection from deleterious agents such as bile and hydrophobic antibiotics (Nikaido and Vaara 1985). *C. jejuni* LPS has been implicated in adherence to human epithelial cells and production of an endotoxin (McSweegan and Walker 1986, Moran 1995). *C. jejuni* LPS may also be involved in invasion/translocation into epithelial cells as has been demonstrated in *E. coli* and *Salmonella typhimurium* (Wallis 1994, Ketley 1995) but the role LPS plays in invasion may be secondary to that of the flagella (Klena *et al* 1998).

LPS is composed of three functional domains. Anchoring the LPS to the outer membrane is a glucosamine-based glycolipid known as lipid A. A non-repeating core oligosaccharide is added at the non-reducing end of lipid A followed by the addition of a distal repeating polysaccharide or O antigen (Raetz 1990, Reeves 1994). *C. jejuni* strains showing the production of a high M_r LPS (with O antigens) e.g., strains HS:19, HS:23 and HS:36 are infrequent. Most strains produce predominantly a low M_r LPS similar to that observed with muscosal bacteria (containing no O antigens). Characterisation of the core oligosaccharides of *C. jejuni* serotypes HS:1, HS:2, HS:3,

HS:4, HS:10, HS:19, HS:23 and HS:36 (Aspinall *et al* 1993a, 1994a, 1994b) reveals variability in linkages and in constituents more closely resembling the lipooligosaccharides (LOS) of *Neisseria* and *Haemophilus* than the enterobacterial LPS (Moran 1995, Preston *et al* 1996).

Research has also implicated the LPS molecule in the development of GBS mentioned previously. Examination of the LPS structure from *C. jejuni* by mass-spectroscopy Aspinall *et al* (1992), found that the terminal regions of core oligosaccharides (OS) of several serotypes of *C. jejuni* mimicked the structures of human gangliosides. Serotypes HS:4 and HS:19 yielded OS structures resembling the G_{M1} and G_{D1a} gangliosides. Serotypes HS:1, HS:23 and HS:36 have shown G_{M2}-like OS structures occur in LPS (Aspinall *et al* 1992). The gangliosides, cell surface components of nerve tissue, are considered the target antigens of antineural antibodies (Yuki *et al* 1994). Sera extracted from GBS patients who previously had *C. jejuni* enteritis contains autoantibodies to G_{M1} gangliosides during the acute phase of illness (Yuki *et al* 1994). *C. jejuni* isolates taken from patients with either GBS or Miller-Fisher syndrome (MFS) contain a characteristic terminal trisaccharide (Neu5Ac(α2-8)Neu5Ac(α2-3)β-D-Galactose) structural feature not found in non-neuropathogenic strains (Salloway *et al* 1996). The presence of *N*-acetyl neuraminic acid may cause increased resistance to human serum but the significance in *C. jejuni* pathogenesis in enteritis cases is uncertain (Salloway 1996).

As mentioned previously the LPS molecule also forms the basis of the heat stable (HS) serotyping scheme of *C. jejuni* with up to 60 serotypes identified (Aspinall *et al* 1993a, Moran 1991). *C. jejuni* LPS has considerable serological heterogeneity despite a relativity low number of carbohydrate antigenic determinants (Beer *et al* 1986, Perez-Perez *et al* 1985). However common to all serotypes surveyed so far is a conserved inner core region composed of 3-deoxy-D-manno-octulosonic acid (KDO) and two heptose residues (Aspinall *et al* 1993b).

The heptose biosynthetic pathway is proposed to be composed of four enzymatic steps (Eidels and Osborn 1971). Figure 3 shows the heptose biosynthetic pathway, illustrating the enzymes and genes involved in the process. Recently, Brooke and

Valvano (1996) provided biochemical evidence for the first enzymatic step. The conversion of sedoheptulose-7-P to D-glycero-D-mannoheptose-7-P is due to phosphoheptose isomerase (glycero-mannoheptose isomerase) in *E. coli*. Upritchard (1997) characterised the *gmhA* gene from *C. jejuni* and *C. coli*. The *waaF* gene, encoding a transferase necessary for the addition of a second heptose residue to the LPS core has also been identified adjacent to, but transcribed in a different orientation from *gmhA* in the strain NCTC 11168 and *C. coli* M275 (Upritchard 1997). Upritchard (1997) observed the *gmhA* gene to be transcriptionally coupled to adjacent genes and physically linked on the chromosome to other genes involved in the biosynthesis and attachment of heptose. For use as an epidemiological marker the *gmhA* gene identified by Upritchard was found to have sufficient heterogeneity between two species examined; 92.7 % identical, 95.1 % similarity of the GmhA protein of *C. coli* M275 versus *C. jejuni* NCTC 11168 protein. Therefore this gene may act as a suitable epidemiological marker. Analysis of the recently submitted NCTC 11168 whole genome sequence has revealed this gene is unlinked to the *flaA* gene[1]. The use of several unlinked genes with sufficient sequence heterogeneity could provide an increased level of discrimination for RFLP analysis.

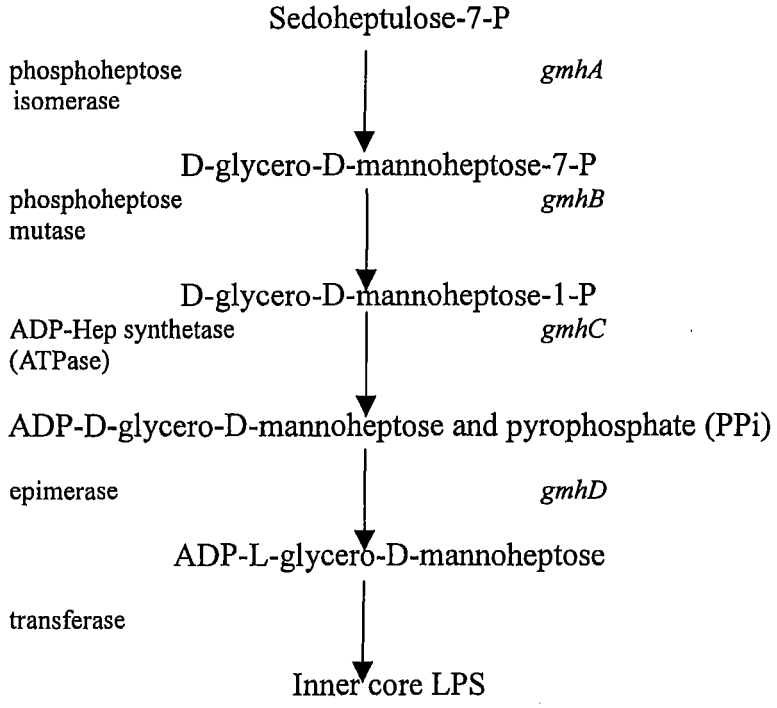


Figure 3: Heptose biosynthesis pathway as proposed by Eidels and Osborn (1971) (adapted from Brooke and Valvano 1996).

[1] The complete NCTC 11168 sequence is available at www.sanger.ac.uk.

1.7 OBJECTIVES OF THIS STUDY.

Members of the *Campylobacter* Genus are important animal and human pathogens (Cover and Blaser 1989, Wallis 1994). *C. jejuni* causes 80-90 % of *Campylobacter* gastroenteritis worldwide (Skirrow 1990). Campylobacteriosis incidence has increased since 1980 in New Zealand, surpassing *Salmonella*, with the incidence in the Canterbury RHA amongst the highest in New Zealand. It is important to understand the relationship of *C. jejuni* strains present within a population. For example, if a particular isolate is observed in a clinical setting and is also observed in an environmental situation, a possible link may be determined. With increased understanding of relatedness and possible sources of infection, mechanisms could be put in place to reduce the number of infections and the seriousness of the disease. An awareness of inappropriate cooking practices may also serve to reduce the number of infections. In identifying the possible sources of infection, rates of campylobacteriosis may decrease. However, most of the cases reported are sporadic, therefore isolating a source is often difficult to achieve.

Typing methods are important in epidemiological studies of *C. jejuni*. Many different systems are available, however, not all are reliable, robust, reproducible and inexpensive. A typing system with a high level of discrimination is needed that can be applied for routine use to analyse large numbers of *C. jejuni*.

The aim of this study was to identify and characterise strains of *C. jejuni* and *C. coli* that were isolated from patients at Christchurch Public Hospital. It was decided to examine the isolates obtained from a hospital environment to determine whether these cases are more severe than those reported in the community. From the literature it was observed that most GBS cases have reported a previous *Campylobacter* infection. Most commonly HS:19 has been the *C. jejuni* strain responsible. By examining the cases of *C. jejuni* and *C. coli* isolated from Christchurch Public Hospital, evidence could be provided as to what strain may have been present in a patient who subsequently developed GBS. GBS cases in New Zealand are rare, with only 1-2 cases reported a year. Due to the rarity of this illness, this study was not able to identify a GBS related isolate. Therefore, this study concentrated on identifying and classifying the strains

present in a hospital environment to determine if they were different from those reported in the community.

To characterise the strains two established techniques were applied. One technique, PCR-RFLP using the *flaA* and *gmhA* genes was used. These two genes are unlinked on the *Campylobacter* genome, therefore providing meaningful discrimination when used in conjunction. The second technique, MLEE, was applied to the largest *flaA* group identified to determine if this subset could be discriminated further.

Concurrently to the clinical characterisation, *C. jejuni* isolated from sheep were also examined by PCR-RFLP of *flaA* and *gmhA*. This was to determine whether there was a clonal difference between sheep (environmental) isolates and those observed in a clinical setting.

This research will also help to establish a database of isolates gathered from different sources, i.e., humans and animals. These results can be added to previous studies which have identified *C. jejuni* from water supplies and a second human source, isolated from non-hospitalised campylobacteriosis sufferers (Armstrong 1997). From this study the relatedness of isolates gathered from hospitalised patients, and those previously observed from gastroenteritis cases (Armstrong 1997), can be compared to determine relatedness and distribution between these environments. In future experiments, results will be able to be compared and added to this database as necessary. Over time, this will increase our knowledge of *Campylobacter* isolates in different environments, ascertaining whether certain *Campylobacter* types are adapted to one environment and whether certain isolates are more virulent than others.

CHAPTER II

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS.

The bacterial strains used in this study are listed in Table 1.

Table 1: List of bacterial strains used in this study.

Isolate Name	Source	Reisolate of	Species
NCTC11168	HF NZRM		*C. jejuni*
XT318V	HF CHL		*C. jejuni*
XV898P	HF CHL		*C. jejuni*
XX158F	HF CHL		*C. jejuni*
YG018S	HF CHL		*C. jejuni*
YG023Q	HF CHL	YG018S	*C. jejuni*
IG022P	HF CHL	YG018S	*C. jejuni*
XM653F	HF CHL		*C. jejuni*
YJ936T	HF CHL		*C. jejuni*
YO808T	HF CHL		*C. jejuni*
YP567F	HF CHL	YO808T	*C. jejuni*
YN438B	HF CHL		*C. jejuni*
YP502F	HF CHL		*C. jejuni*
YU298M	HF CHL		*C. jejuni*
YU304J	HF CHL		*C. jejuni*
BB193H	HF CHL		*C. jejuni*
BL821U	HF CHL		*C. jejuni*
BM271M	HF CHL		*C. jejuni*
BN499U	HF CHL	BM271M	*C. jejuni*
BV374O	HF CHL		*C. jejuni*
CE934Q	HF CHL		*C. jejuni*
CA755I	HF CHL		*C. jejuni*
CI115W	HF CHL		*C. jejuni*
CM123F	HF CHL	BV374O	*C. jejuni*
CU130P	HF CHL		*C. jejuni*
ZB375B	HF CHL		*C. jejuni*
ZB632B	HF CHL		*C. jejuni*
DO399V	HF CHL		*C. jejuni*
DN031T	HF CHL		*C. jejuni*
DO332T	HF CHL	DN031T	*C. jejuni*
DO291I	HF CHL	DN031T	*C. jejuni*
EG546O	HF CHL		*C. jejuni*
EG761C	HF CHL	EG546O	*C. jejuni*
ET973Q	HF CHL		*C. jejuni*
EU369T	HF CHL		*C. jejuni*
EX923G	HF CHL		*C. jejuni*
FD131W	HF CHL		*C. jejuni*
ZF320J	HF CHL		*C. jejuni*
FZ917J	HF CHL		*C. jejuni*
GF923R	HF CHL		*C. jejuni*

Table 1. Continued...

Isolate Name	Source	Reisolate of	Species
ZG339F	HF CHL		*C. jejuni*
CR828J	HF CHL		*C. jejuni*
HI881O	HF CHL		*C. jejuni*
HW961T	HF CHL		*C. jejuni*
ZH604N	HF CHL		*C. coli*
ZH605O	HF CHL	ZH604N	*C. coli*
ZH606P	HF CHL	ZH604N	*C. coli*
ZH641W	HF CHL		*C. jejuni*
ZI814K	HF CHL		*C. jejuni*
JF124L	HF CHL		*C. jejuni*
ZI975L	HF CHL		*C. jejuni*
ZJ271C	HF CHL		*C. jejuni*
ZJ638R	HF CHL		*C. jejuni*
LJ155C	HF CHL		*C. jejuni*
LB445E	HF CHL		*C. jejuni*
LG430P	HF CHL		*C. jejuni*
LG794W	HF CHL		*C. jejuni*
LC667G	HF CHL		*C. jejuni*
LB244Q	HF CHL		*C. jejuni*
KX649A	HF CHL	KZ386I	*C. jejuni*
KZ386I	HF CHL		*C. jejuni*
KV955A	HF CHL		*C. jejuni*
R923	HBCHL		*C. jejuni*
S052	HBCHL		*C. jejuni*
LS897W	HF CHL	LS785M	*C. jejuni*
LS785M	HF CHL		*C. jejuni*
LT038S	HF CHL		*C. jejuni*
LT973B	HF CHL		*C. jejuni*
LR181R	HF CHL		*C. jejuni*
LS466P	HF CHL		*C. jejuni*
MB200L	HB CHL		*C. jejuni*
MB321A	HF CHL	MB200L	*C. jejuni*
MB617S	HF CHL		*C. jejuni*
MD672W	HF CHL		*C. jejuni*
MC715F	HF CHL		*C. jejuni*
MC591P	HF CHL		*C. jejuni*
MS179P	HF CHL		*C. jejuni*
PH790A	HF CHL		*C. jejuni*
PH844R	HF CHL	PH790A	*C. jejuni*
PH845S	HF CHL	PH790A	*C. jejuni*
PF322Q	HF CHL		*C. jejuni*
PD850L	HF CHL		*C. jejuni*
PA268R	HF CHL		*C. jejuni*
MZ347T	HF CHL	PA268R	*C. jejuni*
MM539G	HF CHL		*C. jejuni*
ZN244B	HF CHL		*C. jejuni*
PN728G	HF CHL		*C. jejuni*
ZN474U	HF CHL		*C. jejuni*
QA598G	HF CHL		*C. jejuni*
ZN807W	HF CHL		*C. jejuni*
QZ233U	HF CHL		*C. jejuni*
QP453U	HF CHL		*C. jejuni*
QM664N	HF CHL		*C. jejuni*
RE097T	HF CHL		*C. jejuni*
RF185D	HF CHL		*C. jejuni*
QZ840M	HF CHL		*C. jejuni*

Table 1. Continued...

Isolate Name	Source	Reisolate of	Species
RC703J	HF CHL	QZ233U	*C. jejuni*
RE159O	HF CHL		*C. jejuni*
RC722O	HF CHL		*C. jejuni*
RC317T	HF CHL		*C. jejuni*
QZ550A	HF CHL	QZ840M	*C. jejuni*
ZP028D	HF CHL		*C. jejuni*
QY139W	HF CHL		*C. coli*
RC167B	HF CHL		*C. coli*
RD177L	HF CHL		*C. coli*
CJS-71	Sheep		*C. jejuni*
CJS-72	Sheep		*C. jejuni*
CJS-73	Sheep		*C. jejuni*
CJS-74	Sheep		*C. jejuni*
CJS-75	Sheep		*C. jejuni*
CJS-76	Sheep		*C. jejuni*
CJS-77	Sheep		*C. jejuni*
CJS-78	Sheep		*C. jejuni*
CJS-79	Sheep		*C. jejuni*
CJS-80	Sheep		*C. jejuni*
CJS-81	Sheep		*C. jejuni*
CJS-82	Sheep		*C. jejuni*
CJS-83	Sheep		*C. jejuni*
CJS-84	Sheep		*C. jejuni*
CJS-85	Sheep		*C. jejuni*
CJS-86	Sheep		*C. jejuni*
CJS-87	Sheep		*C. jejuni*
CJS-88	Sheep		*C. jejuni*
CJS-89	Sheep		*C. jejuni*
CJS-90	Sheep		*C. jejuni*

HF CHL = Human Faeces Canterbury Health Laboratories.
HB CHL = Human Blood Canterbury Health Laboratories.
HF NZRM = Human Faeces, New Zealand Reference Material.
CJS = *Campylobacter jejuni* sheep.

2.1.1 SOURCE OF ISOLATES.

Isolates were collected from Canterbury Health Laboratories (CHL) from March 1997 until March 1998. *Campylobacter jejuni* was mainly isolated from stool samples of patients who presented at Christchurch Public Hospital with gastrointestinal illness. However, three isolates were obtained from blood samples taken from patients who presented to Christchurch Public Hospital with bacteremia during this period. *Campylobacter jejuni* and *Campylobacter coli* isolates were initially cultured from stool/blood on *Campylobacter* Charcoal Differential Agar (CCDA, Oxoid) and incubated in the laboratory at 42°C in an atmosphere containing 10 % CO_2 for 48 h. Dr Stan Fenwick (Massey University) provided twenty *C. jejuni* isolates from

slaughtered sheep.

2.2 BUFFERS AND MEDIA.

Solutions and media used in this study were prepared as described in appendix 1.

2.2.1 ANTIBIOTICS.

Cefoperazone (Cp) was added to molten CCDA media in order to select for *Campylobacter*. Cp was added to a final concentration of 32 μg/ml in this study.

2.3 BACTERIOLOGICAL METHODS.

2.3.1 CULTURE CONDITIONS.

Campylobacter jejuni and *C. coli* isolates were incubated in a water-jacketed CO_2 incubator (Nuaire) in a microaerophilic environment of 10 % CO_2. Incubation temperatures were either 37°C or 42°C and solid media (CCDA with the addition of Cp or Mueller-Hinton Agar containing 5 % defibrinated sheep's blood) was used.

2.3.2 IDENTIFICATION OF *C. jejuni* AND *C. coli.*

Moist, flat, grey, spreading colonies visible on the CCDA media were presumptively identified as *C. jejuni*. The 48 h old bacterial colonies were Gram-stained to visualise the organisms. Nalidixic acid sensitivity was used to distinguish different *Campylobacter* spp. (Tenover *et al* 1992). Nalidixic acid sensitive isolates were examined using hippurate hydrolysis. Differentiation of *C. jejuni* from *C. coli* relies on the ability of *C. jejuni* to hydrolyse hippurate (Linton *et al* 1997). Gram-negative, nalidixic acid sensitive and hippurate positive organisms were considered to be *C. jejuni* (Nachamkin 1992).

2.3.3 STORAGE OF STRAINS.

For day to day use isolates were maintained on CCDA plates supplemented with Cp at 37°C or 42°C in an atmosphere containing 10 % CO_2. Long term storage of isolates was achieved by harvesting bacterial growth from CCDA plates using Brain Heart Infusion broth (Gibco BRL) containing sterile glycerol (BDH) (final concentration of

20 %) using a sterile glass rod. A 1.5 ml aliquot was mixed with 6-8 (2.5 to 3.5mm dia.) sterile glass beads (BDH) contained in Nunc cryotubes. Aliquots of isolates were stored at -80°C until required.

2.4 ANALYTICAL TECHNIQUES.

2.4.1 POLYMERASE CHAIN REACTION.

Polymerase chain reactions (PCR) of bacterial DNA were performed using a Corbett Research FTS-320 Thermal Sequencer. *Taq* polymerase and buffers were purchased from Qiagen, dNTPs were purchased from Gibco-BRL and primers (Table 2) were purchased from Amrad-Pharmacia Biotech.

Table 2: PCR Primers.

Primer	Nucleotide Sequence	Locus	Organism
94.293	5'-GCA ATA AAA CCA CTA TCA-3'	*waaF*	*C. jejuni*
96.01	5'-CAA GAG TTG AAT TGA TCG-3'	*gmhA*	*C. jejuni*
96.11 (pg 50)	5'-ATG GGA TTT CGT ATT AAC-3'	*flaA*	*C. jejuni*
96.17 (NR 2)	5'-CTG TAG TAA TCT TAA AAC ATT TTG-3'	*flaA*	*C. jejuni*

Each PCR consisted of several reagents added to a thin-walled 0.5 ml Eppendorf tube (all concentrations are final for 100 µl reactions): 3 pmol/µl of each primer (forward and reverse), 250 µM dNTP (dGTP, dATP, dTTP, dCTP), 3 mM $MgCl_2$, and sterile ddH_2O. Using a sterile pipette tip, a bacterial colony was removed from a CCDA plate and added to a mixture containing the above reagents (89.5 µl). The reactants were placed in the thermal sequencer and DNA templates were denatured by incubation at 99°C for 10 min. Tubes were briefly cooled to ambient temperature by incubation at 25°C for 1 minute. Mixtures were microfuged for 5 seconds at 13,400 x g before the addition of 10 x PCR buffer (containing 15 mM Mg^{2+}) and 2.5 U *Taq* polymerase, bringing the final volume of each reaction to 100 µl. Mixtures were briefly pulsed in the centrifuge to collect all components of the reaction. Reaction mixtures were overlaid with 100 µl of sterile paraffin oil and returned to the thermal sequencer. PCR amplification consisted of the following cycling conditions: 94°C for 1 min to denature

template followed by 35 cycles of 94°C for 15 sec, an annealing step of 55°C for 45 sec, and extension of templates at 72°C for 1 min and 45 sec (Nachamkin 1996). A 36th cycle in which the denaturing/annealing conditions were the same, except the final cycle was extended to 5 min at 72°C to allow for completion of all initiated products was added. PCR products were removed from under the paraffin oil, transferred to a sterile 1.5 ml tube and stored at -20°C. Control reactions containing all reagents except bacterial DNA served as a negative control. *C. jejuni* strain NCTC 11168, known to reliably produce an amplicon with the chosen primers, was used for a positive control. Control reactions were performed with every PCR procedure.

2.4.2 RESTRICTION FRAGMENT LENGTH POLYMORPHISMS.

The restriction endonuclease *Dde*I (Boehringer Mannheim) was used to detect restriction fragment length polymorphisms (RFLP) in genes amplified from *C. jejuni* isolates. *flaA* and *gmhA* PCR amplicons generated by the described PCR conditions, were examined by *Dde*I digestion and electrophoresis to determine their respective RFLP profiles. In all cases the PCR generated amplicon was used directly for RFLP analysis, no clean up procedure was performed. Restriction endonuclease digestion reactions were carried out according to the manufacturer's protocol using buffers supplied. Reactions were performed in a final volume of 15 µl and incubated at 37°C for 3 hours. The extent of restriction endonuclease digestion was determined by agarose gel electrophoresis, followed by staining of DNA fragments with ethidium bromide, and visualisation using a UV transilluminator (302 nm).

2.5 AGAROSE GEL ELECTROPHORESIS OF DNA.

Amplicons from PCR reactions and amplicons modified by restriction endonuclease digestions were separated by Tris-acetate-EDTA (TAE) buffered agarose gel electrophoresis. Agarose was dissolved in 1 x TAE buffer (appendix 2) to give a final agarose concentration ranging from 0.8 % (to resolve high molecular weight DNA fragments) to 4 % (for resolution of DNA fragments <0.5 kb, generated by *Dde*I digestion). DNA samples were mixed with 2 µl of 6 x gel loading buffer (appendix 2) before loading into a preformed agarose well. PCR-RFLP amplicons were separated using an electric field of 50-100 volts for 1-2.5 hr in either a mini-sub (Bio-Rad) or

submarine electrophoresis cell (Jordan) apparatus, connected to either a Model 1,000/500 (Bio-Rad) or a Mighty-Slim 5 X 250 (Hoefer) power supply. DNA was stained with ethidium bromide (0.5 μg/ml) in 1 x TAE for 15-20 min. DNA fragments were visualised using a Sigma T2210 UV (302 nm) transilluminator or an Ultra-Lum KS-3000 Visualisation-Documentation and Analysis system (Ultra-Lum, Carson, CA).

2.6 ANALYSIS OF RFLP PATTERNS.

PCR-RFLP patterns were photographed using Polaroid film (Black and white instant pack film 667) to allow for permanent storage and comparison. Alignment and comparison of patterns was performed manually. When undertaking RFLP experiments, NZRM 1958 (NCTC 11168) was used as a positive control to ensure reproducibility between experiments. This also enabled gel comparisons between each experiment. When an isolate provided a profile similar to that of another isolate, both isolates were subjected to RFLP analyses again and visualised on an agarose gel. This technique demonstrated whether the isolates were identical or if a variation existed, therefore allowing classification into different RFLP profiles. This procedure allowed accurate determinations to be made of the isolates examined using PCR-RFLP. The same molecular weight marker (100 bp DNA ladder, Promega) was used in each experiment. On the basis of visual comparisons each RFLP profile was assigned to a particular *flaA* or *gmhA* classification.

2.7 MULTILOCUS ENZYME ELECTROPHORESIS.

2.7.1 BUFFERS AND ENZYMES.

The buffers and enzymes used for multilocus enzyme electrophoresis in this study are listed in appendix 3.

2.7.2 ISOLATE PREPARATION.

Fifteen clinical strains of *C. jejuni* (based on their *flaA* classification) were cultured on CCDA at 42°C in 10 % CO_2, for 48 h. A single colony from each strain was restreaked onto Nutrient Broth No. 2 agar (appendix 1) and cultured at 42°C, 10 % CO_2, for 48 h. After this time 30 ml Nutrient Broth No. 2 (appendix 1) was inoculated with a bacterial

suspension taken from the plate using a sterile 1 µl plastic loop and incubated at 42°C, in 10 % CO_2 for 48 h. The entire bacterial culture (estimated to contain 2 x 10^8 cfu/ml as determined by serial plate counts) was transferred to 30 ml Oakridge centrifuge tubes (Nalgene) and centrifuged at 13,800 x g, 4°C for 10 min. The bacterial pellet was resuspended in 1 ml of extraction buffer (appendix 3), and transferred to a sterile 2 ml polypropylene tube. The bacterial suspension was mixed with 2 g of 0.1 mm Zirconium silicate beads (BioSpec Products) and shaken at 5,000 rpm for 30 sec in a Mini Beadbeater Cell Disrupter (BioSpec Products). Samples were incubated on ice until all isolates had been lysed; the procedure was repeated to ensure complete lysis. The polypropylene tubes were pulsed briefly (<5 sec) in a microfuge to collect the silicate beads. Supernatants were transferred to a sterile 1.5 ml Eppendorf tube and centrifuged at 6,600 x g, 4°C, for 3 min. Cell lysates were stored at -80°C until required.

2.7.3 MLEE ELECTROPHORETIC TECHNIQUE.

TG Buffer (appendix 3) was loaded into each well of a Helena electrophoresis apparatus (Helena Laboratories, Texas, USA). A filter paper wick was placed over the edges of the inner region of the electrophoresis apparatus ensuring it was immersed in the tank buffer. Cellulose acetate plates (7.5 cm x 7.5 cm) were soaked in TG Buffer (appendix 3) and incubated at ambient temperature for 15-20 min. A 10 µl aliquot of each sample was loaded into a sample well of the sample loading plate (maximum 12 samples). After blot drying briefly with Whatman 3 mm filter paper, bacterial lysates were applied to the cellulose acetate plate using a Super Z-12 applicator supplied by the manufacturer. An aligning base was used to assist sample loading onto the cellulose acetate plate. Plates were placed face down onto the filter paper wick to ensure connection. The electrophoresis apparatus was connected to a Mighty-Slim 5 X 250 (Hoefer) power supply. Undenatured enzymes were separated by exposure to an electric field of 100-200 volts for 20-25 mins; 6 x agarose gel buffer served as an indicator of enzyme migration. When the blue indicator dye was 1 cm from the cathode, electrophoresis was discontinued and cellulose acetate plates were stained immediately in the appropriate detection buffer (appendix 3) and incubated at 37°C for 20-30 mins to allow visualisation of the colourimetric product.

2.7.4 MLEE DETECTION.

To enhance enzyme detection numerous variations of the experimental procedure were performed. Experimentation with the pH of TG Buffer was investigated; pH values of 7.0, 7.5 8.0 and 8.5 were trialled to determine the optimum pH for the buffer system. Both the buffer that the cellulose acetate plates soaked in and the electrode buffer were of the same pH. Four enzymes were trialled during this protocol. These were malate dehydrogenase $NADP^+$ (ME), malate dehydrogenase NAD^+ (MDH), phenyalanine–leucine peptidase (PEP) and catalase (CAT) (appendix 3).

In addition to varying pH, enzymes were detected with or without an agar overlay (appendix 3) (Selander *et al* 1986, Hebert and Beaton 1989). Initially the agar was applied to the cellulose acetate plate following application of the desired stain to the plate with poor results. The second attempt was to apply the agar to the cellulose acetate plate prior to the stain being applied; again with poor results. The agar was incorporated into the desired stain (generally equal volumes), mixed briefly, and applied to the cellulose acetate plate before hardening of the agar. This third procedure was found to yield the best results and therefore used for subsequent experiments.

Staining for ME, MDH and PEP was carried out at 37°C for 20 min. If no bands were evident after the initial 20 min, the cellulose acetate plate was incubated for an additional 10 min. If no bands were evident after this time a negative result was recorded. The cellulose acetate plates were rinsed for 20 min in H_2O to remove the agar overlay, and a photo taken. Cellulose acetate plates were allowed to air dry at ambient temperatures or were placed in an oven at 55°C for 10 min. Plates could be rehydrated with H_2O for future analysis.

Staining for CAT was always performed at ambient temperature. Initial electrophoretic conditions were as described above, however it was found that at 100 V, the migration of the samples was uneven and samples did not migrate as quickly as during the previous experiments. Due to slow enzyme migration the electric current was discontinued when the blue indicator dye was half way along the plate. Staining was as described by Aeschbacher and Piffaretti (1989). Briefly, plates were incubated at ambient temperature in a 50 ml solution of 50 % (w/v) sodium sulfite and hydrogen

peroxide for 15 min, followed by a brief rinse with distilled H_2O. To stain for catalase, cellulose acetate plates were immersed in freshly made 1.5 % (w/v) potassium iodide and agitated gently. Plates were removed from the staining solution when white zones appeared on a dark blue background.

2.7.5 INTERPRETATION AND SCORING OF ALLELES.

Scoring of alleles on the cellulose acetate plates was performed by visually assigning the slowest migrating band as a 1; the next slowest as a 2 and so on. If a particular strain had two alleles it was assigned two numbers. For example, the slowest migrating allele a 1 and the fastest migrating allele a 4.

2.8 ANALYSIS OF PATTERNS.

2.8.1 S-PLUS.

Profiles generated by PCR-RFLP and MLEE were analysed using the S-plus package (appendix 4) and a dendrogram showing relatedness of isolates generated. For PCR-RFLP each band was assigned an allele in order of migration, with the slowest migrating band being designated a 1, until all bands had been assigned (appendix 5). Bands that were under 100 bp were not assigned a number as separation at this point was difficult to visualise. The profiles were then applied to the statistical package and a dendrogram generated.

2.8.2 DISCRIMINATION VALUE.

The level of discrimination was calculated for all typing methods, to determine the effectiveness of these methods as epidemiological markers. The D value was calculated using Simpson's index of diversity:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

Where N is the total number of strains in the sample size; s is the total number of types described and n_j is the number of strains belonging to the j^{th} type (Hunter and Gaston 1988).

CHAPTER III

RESULTS

3.1 CLINICAL ISOLATE INFORMATION.

In this study 105 isolates of *Campylobacter* spp. obtained from human faeces or blood were examined. Isolates were collected from Canterbury Health Laboratories (CHL) from March 1997 to March 1998. Figure 4 shows the number of isolates collected each month as part of this study relative to the number of *Campylobacter* infections reported to the Southern Regional Health Authority (SRHA). Overall 10.8 % of the campylobacteriosis cases reported were examined during this study. The sample size of this study was limited to patients who were hospitalised in Christchurch and from the South Canterbury region. A majority of the cases reported to the SRHA would not have been admitted to hospital and did not fit the scope of this investigation.

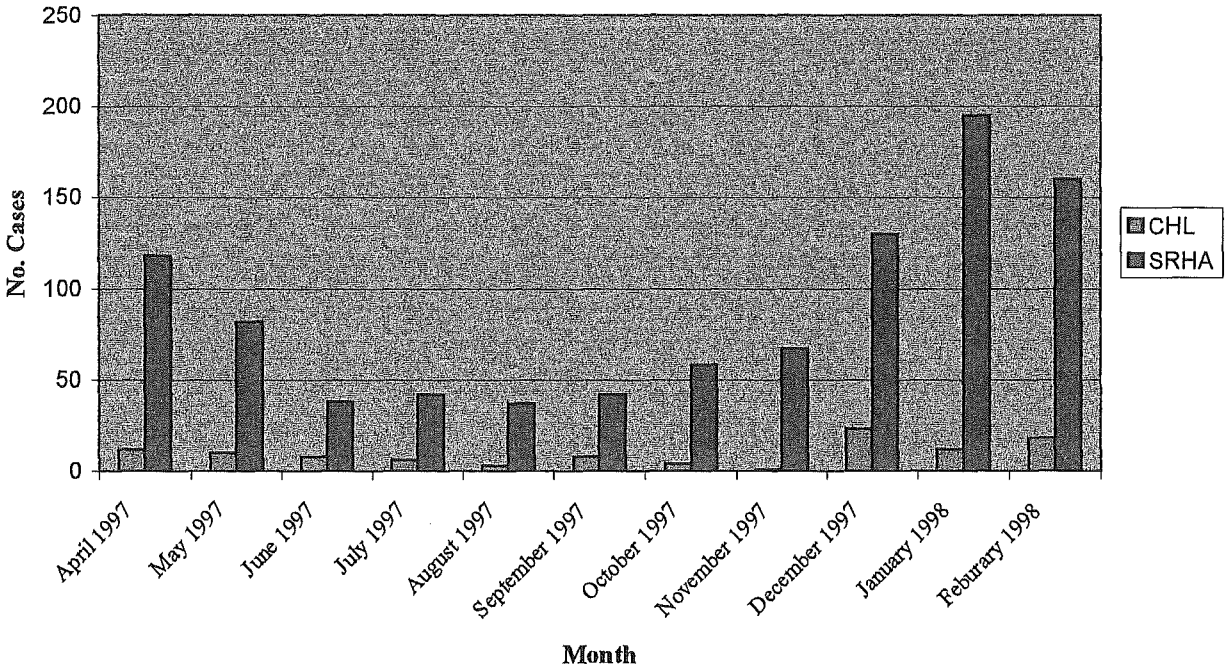


Figure 4: *Campylobacter* infections in the Canterbury region 1997-98.
CHL = Canterbury Health Laboratories.
SRHA = Southern Regional Health Authority.

Of the 105 clinical isolates three (2.9 %) were isolated from the blood of patients, the remaining 97.1 % were obtained from faeces. In this study six isolates (5.7 %) were identified as *C. coli* while the remaining 94.3 % were classified as *C. jejuni*. The identification of isolates to species level was performed by Gram-staining, nalidixic acid sensitivity and hippurate hydrolysis.

In this study 84 patients were examined. These patients provided 105 isolates with 15 providing multiple isolates during the course of illness, either 2 or 3 samples. Table 1 (Chapter 2, p 23) shows the relationship of isolates to one another, listing the reisolated samples. Demographic data relating to age, sex and geographic location was collected (appendix 5). For one patient no information was provided. Of the remaining 83 patients, 64.3 % (n=54) were males, while 34.5 % (n=29) were females. This study supports the observations reported in the literature that males are more prominent in suffering from campylobacteriosis than females.

There are two dominant age groups that suffer from campylobacteriosis. These are 1-4 years and 20-29 years of age. From this study it was observed that 20-29 year olds represented the largest number of infections (n=27), with males representing 19 cases. However the 1-4 age group did not follow this trend, with only five cases of which males accounted for four. This study showed the age group 30-39 years to have the second largest number of infections, with 16. This group was evenly distributed between males and females, each having eight cases. Table 3 shows the distribution of cases into age and sex (n=84).

Table 3: Distribution of patients according to age and sex.

age	<1	1-4	5-9	10-14	15-19	20-29	30-39	40-49	50-59	60-69	70-79	80+
male	3	4	0	6	2	19	8	2	3	3	2	2
female	0	1	1	0	3	8	8	1	0	2	3	2
total	3	5	1	6	5	27	16	3	3	5	5	4

The majority of these patients were admitted to Christchurch Public Hospital (n=52), with one patient being an overseas traveller. Patients from the Ashburton region reported the second largest number of cases in this study. No information was gathered as to a possible source of infection for these patients. It has been assumed that these

cases were sporadic, therefore proving difficult to pinpoint a source. *C. jejuni* isolate MB200L, isolated from blood, also had a correlating sample taken from the patient's faeces, MB321A. This patient was a 23-year-old male, admitted to Christchurch Public Hospital. It is likely that the patient suffered from a severe case of gastroenteritis, and/or may have suffered complications. The other two blood samples, R923 and S052, were isolated from the blood of two other patients. R923 was an 80 year old male, while S052 was a 39 year old female. Both patients were from Christchurch, providing an isolate only from blood.

3.2 CLINICAL ISOLATES: *flaA* PCR-RFLP ANALYSIS.

The 105 clinical isolates were examined by PCR-RFLP analysis, using primers specific for the flagellin gene *flaA* (Nachamkin *et al* 1993). 88 of 105 isolates (83.8 %) generated the expected 1.7 kb amplicon. This represented 72 of the patients, including a patient who had provided three isolates. However only one of these isolates generated the expected 1.7 kb amplicon. Therefore this patient is also represented in the group who did not provide a result (see below). Isolates that did not provide an amplicon were retested 2 to 3 times in total, with no result. Annealing temperatures were altered to 50°C in an attempt to generate the 1.7 kb amplicon, without success. This sample consisted of 13 patients, with two patients providing multiple isolates, and the remaining only providing one isolate. One patient had provided three samples, of which two (YG018S and YG022Q) did not generate a PCR amplicon, and these were included in this group. The other patient who provided three isolates did not generate a *flaA* amplicon with any of the isolates (PH790A, PH844R and PH845S). Methodology using purified DNA was not attempted as a template for PCR analysis.

All experimental procedures used a positive *C. jejuni* control strain, NCTC 11168. NCTC 11168 resulted in a 1.7 kb amplicon in repetitive trials under the experimental conditions. A negative control containing all reagents except a DNA template was used and this repeatedly provided no evidence of amplification or contamination. Incorporating a positive and negative control provided confidence that the experimental conditions had remained the same. Typical results of *flaA*-PCR conducted on isolates are shown in figure 5.

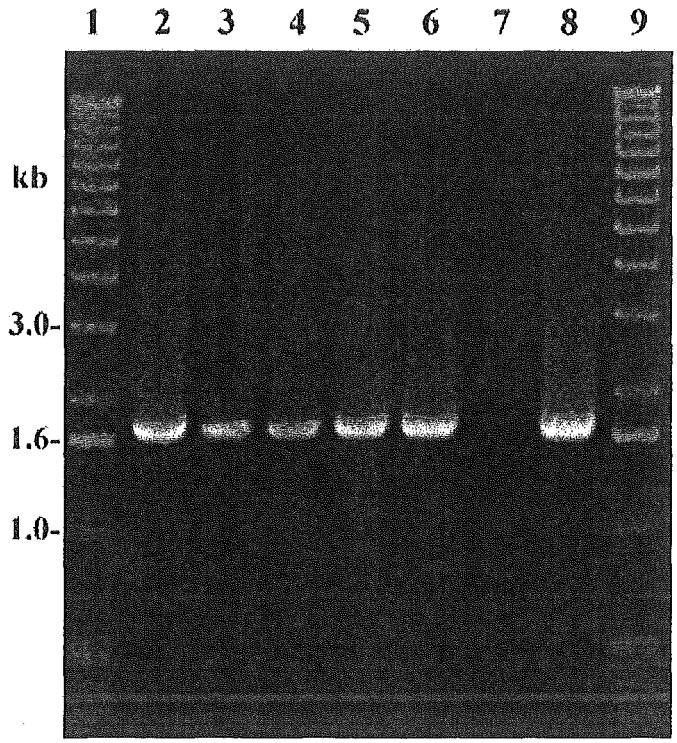


Figure 5: Agarose gel showing PCR amplicons generated by primers pg 50 and NR 2. PCR amplicons were electrophoresed through a 0.8 % agarose gel. Lane 1 and 9, 1 kb DNA ladder (Gibco BRL); Lane 2, BL821U; Lane 3, ET973Q; Lane 4, IG022P; Lane 5, CA755I; Lane 6, DN031T; Lane 7, negative control; Lane 8, NCTC 11168.

flaA-PCR positive isolates (n=88) were restriction digested using the enzyme *Dde*I to generate an RFLP profile. Of these 88 isolates, 87 provided a measurable profile (83 % of the original sample). Table 4 summarises the *flaA* types generated in this study.

Figure 6 is a physical map of the *flaA* gene from NCTC 11168, showing the location of *flaA* primers pg 50 and NR 2. Also illustrated on this physical map are the two conserved regions (C_1 and C_2) flanking the variable region (V_1). The *Dde*I digestion sites are indicated throughout the gene.

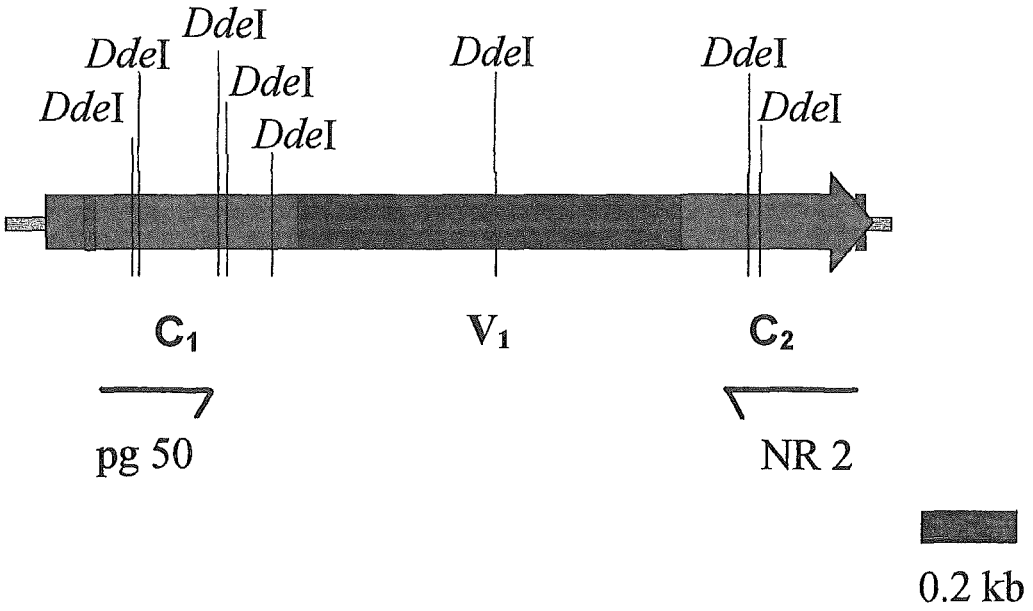


Figure 6: Physical map of NCTC 11168. The PCR amplicon generated using *flaA* primers pg 50 and NR 2; *Dde*I digestion sites, conserved (C_1 and C_2) and variable (V_1) regions are shown.

Table 4: Summary of isolates examined in this study, including *flaA* and *gmhA* classifications.

Isolate Name	Source	*flaA* type	*gmhA* type
LB244Q	HF CHL	1	NB
QZ233U	HF CHL	1	NB
JF124L	HF CHL	2	1
ZF320J	HF CHL	2	2
PF322Q	HF CHL	2	2
ZN474U	HF CHL	2	2
ZN807W	HF CHL	2	2
RE159O	HF CHL	2	2
EG546O	HF CHL	2	5
EG761C	HF CHL	2	5
QM664N	HF CHL	2	7
QZ840M	HF CHL	2	7
QZ550A	HF CHL	2	7
LJ155C	HF CHL	3	4
ET973Q	HF CHL	3	5
EX923G	HF CHL	3	5
FD131W	HF CHL	3	5
YU304J	HF CHL	3	NB
MB617S	HF CHL	4	3
EU369T	HF CHL	4	6
KX649A	HF CHL	4	7
KZ386I	HF CHL	4	7
GF923R	HF CHL	5	6
CR828J	HF CHL	5	6
ZG339F	HF CHL	5	NB
ZJ271C	HF CHL	5	NB
NCTC11168	HF NZRM	6	1
BL821U	HF CHL	6	1
CE934Q	HF CHL	6	1
CA755I	HF CHL	6	1
R923	HBCHL	6	1
S052	HBCHL	6	1
LT038S	HF CHL	6	1
LS466P	HF CHL	6	1
MB200L	HB CHL	6	1
MB321A	HF CHL	6	1
QP453U	HF CHL	6	1
RE097T	HF CHL	6	1
RF185D	HF CHL	6	1
RC722O	HF CHL	6	1
ZB632B	HF CHL	6	4
ZP028D	HF CHL	7	3
RC167B*	HF CHL	7	3
LB445E	HF CHL	7	NB
ZB375B	HF CHL	9	2
IG022P	HF CHL	9	6

Table 4: Continued...

Isolate Name	Source	*flaA* type	*gmhA* type
QY139W*	HF CHL	9	F
RD177L*	HF CHL	9	F
XV898P	HF CHL	9	NB
YO808T	HF CHL	9	NB
YP567F	HF CHL	9	NB
LC667G	HF CHL	13	NB
YN438B	HF CHL	14	1
YU298M	HF CHL	14	1
HI881O	HF CHL	14	1
LG430I	HF CHL	14	3
PA268R	HF CHL	14	NB
MZ347T	HF CHL	14	NB
RC703J	HF CHL	14	NB
XX158F	HF CHL	16	1
XM653F	HF CHL	16	1
BM271M	HF CHL	16	1
BN499U	HF CHL	16	1
BV374O	HF CHL	16	1
CM123F	HF CHL	16	1
CU130P	HF CHL	16	1
HW961T	HF CHL	16	1
RC317T	HF CHL	16	1
XT318V	HF CHL	16	NB
YP502F	HF CHL	21	3
BB193H	HF CHL	21	7
CI115W	HF CHL	21	NB
DO399V	HF CHL	21	NB
LG794W	HF CHL	21	NB
DN031T	HF CHL	22	5
DO332T	HF CHL	22	5
DO291I	HF CHL	22	5
LS897W	HF CHL	23	4
LS785M	HF CHL	23	4
LT973B	HF CHL	23	4
LR181R	HF CHL	23	4
MM539G	HF CHL	23	NB
ZI814K	HF CHL	24	1
ZH604N*	HF CHL	25	NB
ZH605O*	HF CHL	25	NB
ZH606P*	HF CHL	25	NB
ZH641W	HF CHL	26	1
ZI975L	HF CHL	NB	1
MD672W	HF CHL	NB	4
MC715F	HF CHL	NB	4
MC591P	HF CHL	NB	4
YG018S	HF CHL	NB	NB
YG023Q	HF CHL	NB	NB

Table 4: Continued...

Isolate Name	Source	*flaA* type	*gmhA* type
YJ936T	HF CHL	NB	NB
FZ917J	HF CHL	NB	NB
ZJ638R	HF CHL	NB	NB
KV955A	HF CHL	NB	NB
MS179P	HF CHL	NB	NB
PH790A	HF CHL	NB	NB
PH844R	HF CHL	NB	NB
PH845S	HF CHL	NB	NB
PD850L	HF CHL	NB	NB
ZN244B	HF CHL	NB	NB
PN728G	HF CHL	NB	NB
QA598G	HF CHL	NB	NB

HF CHL = Human Faeces Canterbury Health Laboratories.
HB CHL = Human Blood Canterbury Health Laboratories.
F = Faint RFLP pattern, not distinguishable.
NB = No PCR Band.
HF NZRM = Human Faeces, New Zealand Reference Material.
* = *C. coli* isolates, as determined by hippurate hydrolysis.

Using *Dde*I 17 different *flaA*-RFLP profiles were generated. The profiles were characterised based on a previous study (Armstrong 1997), not as described by Nachamkin. RFLP profiles that were not observed in this previous study were assigned a new number in order of observation. For example *flaA* profiles 21 to 26 were new RFLP profiles to this study, the other profiles were observed previously. *flaA* profiles generated in this study are shown in figure 7. *flaA* profiles were not evenly represented in this study. Figure 8 shows the distribution of the *Campylobacter* isolates by *flaA* profiles.

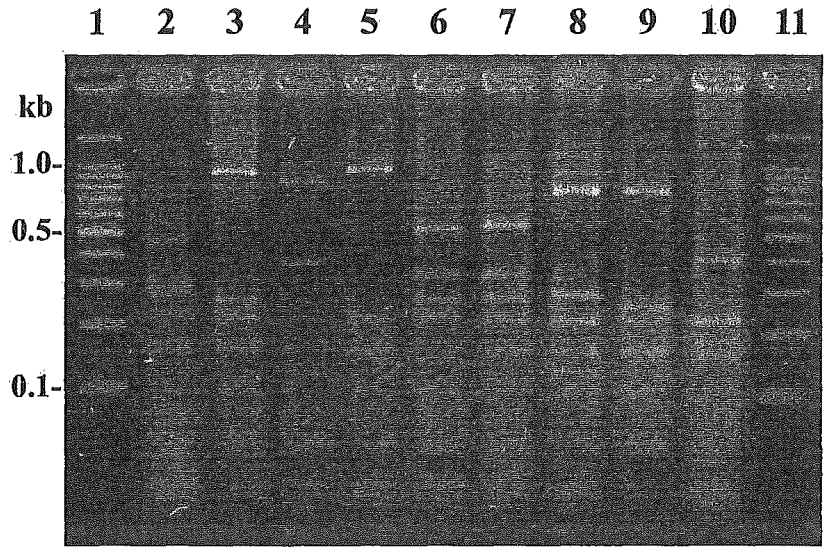
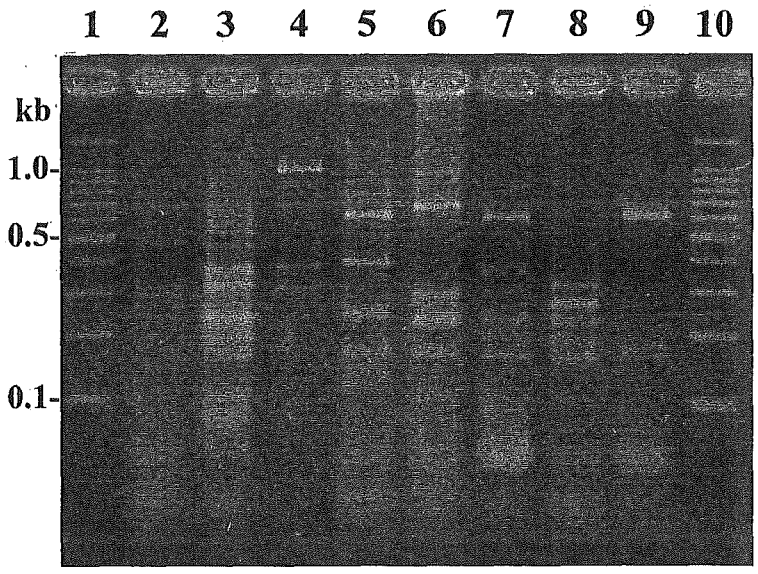


Figure 7: *Dde*I restriction endonuclease digests of representative *flaA* PCR amplicons. Digested amplicons were analysed by electrophoresis through a 4 % agarose gel. **Panel A:** Lanes 1 and 11, 100 bp DNA ladder (Promega); Lane 2, *flaA* 1 QO233U; Lane 3, *flaA* 2 ZF320J; Lane 4, *flaA* 3 FD131W; Lane 5, *flaA* 4 EU369T; Lane 6, *flaA* 5 CR828J; Lane 7, *flaA* 6 NCTC 11168; Lane 8, *flaA* 7 RC167B; Lane 9 *flaA* 9 IG022P; Lane 10, *flaA* 13 LC667G.



Panel B: Lanes 1 and 10, 100 bp DNA ladder (Promega); Lane 2, *flaA* 14 RC703J; Lane 3, *flaA* 16 BN499U; Lane 4, *flaA* 21 BB193H; Lane 5, *flaA* 22 DO399V; Lane 6, *flaA* 23 MM539G; Lane 7, *flaA* 24 ZI814K; Lane 8, *flaA* 25 ZH606P; Lane 9, *flaA* 26 ZH641W.

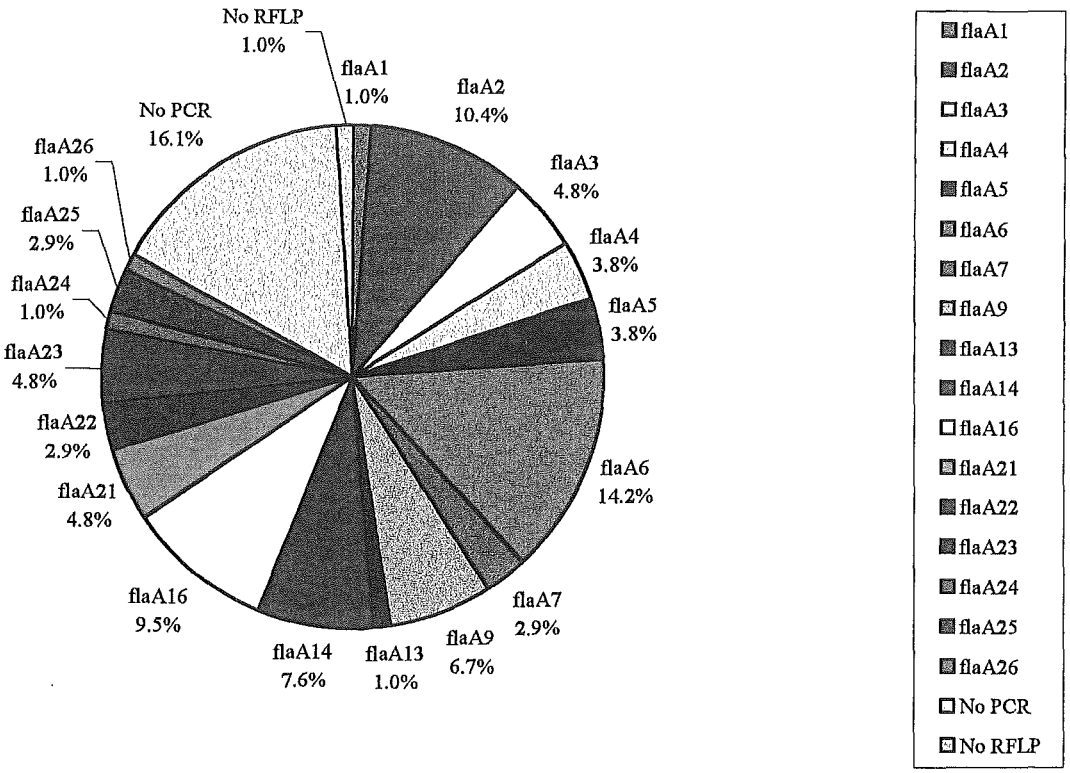


Figure 8: Distribution of clinical isolates into *flaA* classifications.

The largest *flaA* group determined in this study was type 6, with 15 isolates (14.2 % of the typeable isolates). This group also included NCTC 11168. *flaA* group 2 contained 11 isolates (10.4 % of the typeable isolates), followed by *flaA* 16 with 9.5 % (n=10). In contrast Armstrong (1997) observed *flaA* 1, with seven out of 41 isolates to be the largest group. Of the *flaA* profiles that were new to this study (ie., 21 to 26) groups 21 and 23 were the most commonly observed, with 5 isolates (4.8 %) in each. However 17 out of the 105 isolates were non-typeable, representing the overall largest group.

In order to analyse the *Dde*I-RFLP results, DNA fragments were awarded a 1 or 0 according to the absence or presence of a band at a certain migration distance from the well origin. The data generated by these profiles was converted to a dendrogram, to show the relatedness between different *flaA* groups (figure 9). The x-axis represents percent genetic similarity, with 1.0 indicating identical, and 0.0 indicating completely unrelated. Figure 9 shows that the two most closely related *flaA* groups are 2 and 9 (70 % similar). At the 30 % similar mark on the dendrogram, seven clusters are observed. The clusters are represented by *flaA* 3, 21, 22 and 26, which appear to be unrelated to the majority of isolates examined. The main body of isolates are further

divided into three main clusters, represented by *flaA* 5 and 14, and the main cluster consisting of the remainder of isolates. The main cluster consists of three smaller clusters, with *flaA* 2, 4 and 9 in one group, *flaA* 13, 16 and 24 in a second group and *flaA* 1, 6, 7 and 23 in the third.

The information presented in the dendrogram suggests isolates within the closely related cluster have a recent common ancestor. *flaA* types that do not fall into this main cluster, *flaA* 3, 5, 14, 21, 22 and 26, possibly deviated from the most common *flaA* ancestral allele some time ago. The dendrogram generated in this study shows the heterogeneity within *flaA* alleles.

Simpson's index of diversity was used to determine whether the *flaA* typing method was discriminative enough to use as an epidemiological test. According to Hunter and Gaston (1988) the higher the D value, the increased chance that a new isolate will be able to be classified into an already existing profile. Using the total number of isolates that provided an RFLP profile (n=87) a D value of 92 % was calculated. Whereas a value of 95 % or better would be ideal showing 95 % confidence, *flaA* typing provides a level of discrimination adequate to characterise a new isolate. If a D value is calculated using the original data set of 105 isolates, 94 % of the time new isolates could be classified into an existing category. While this value is higher than when including all isolates a large portion of the isolates were unable to provide a *flaA* amplicon, and may therefore be misleading.

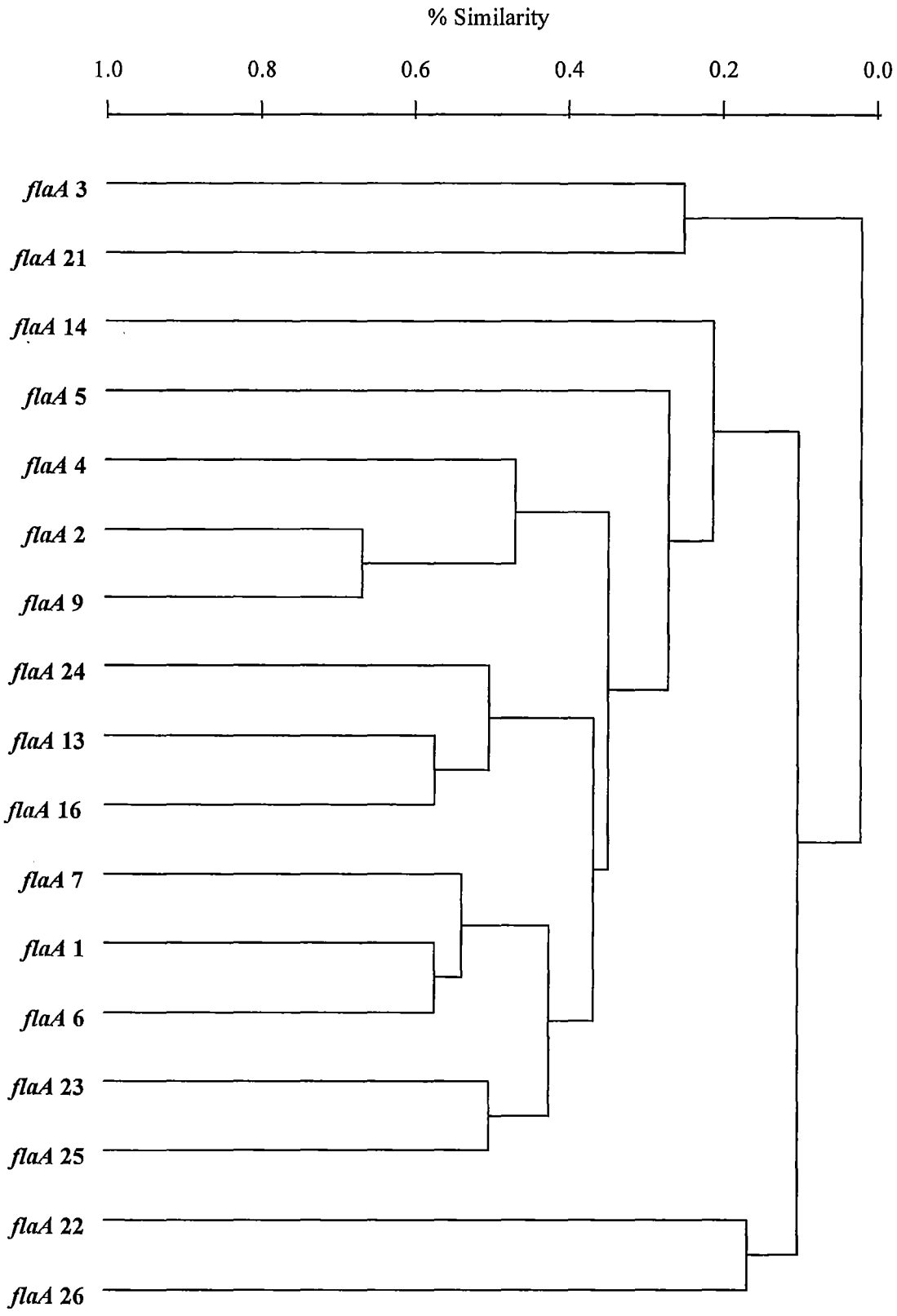


Figure 9: Dendrogram illustrating the relatedness of clinical isolates using the *flaA* gene.

3.3 CLINICAL ISOLATES: *gmhA* PCR-RFLP ANALYSIS.

The 105 clinical isolates previously examined for *flaA* were used to determine the presence of the *gmhA* gene. Using primers 96.01 and 94.293, *gmhA* and a portion of *waaF* were amplified (Upritchard 1997). Of the 105 isolates 75 (71.4 % of the sample size) provided an amplicon of either 900 bp or 1.6 kb. The presence of a 1.6 kb amplicon indicated that these isolates (YP502F, LG430I, MB617S, ZP028D and RC167B) were likely to possess another gene in addition to *gmhA* and *waaF*. These isolates were classified on the basis of their *Dde*I-RFLP profile as *gmhA* 3. *gmhA* 3 has been observed in both *C. jejuni* and *C. coli* isolates during this study, therefore does not appear to be a characteristic of a particular species. Six isolates failed to generate either a 900 bp or 1.6 kb band, but consistently provided multiple bands. This group was not subjected to RFLP procedures, as the presence of multiple bands would have made interpretation difficult. These isolates were designated *gmhA* 7. The 900 bp isolates were classified according to the RFLP profile provided.

In all the experimental procedures NCTC 11168 was used as a positive control, repeatedly generating the 900 bp amplicon. A negative control containing all the reagents except DNA template was used, this repeatedly provided no evidence of an amplicon or contamination.

gmhA-positive PCR amplicons (n=68) were restriction digested using *Dde*I to generate an RFLP profile. 66 isolates (62.9 % of the original sample) provided a distinguishable RFLP profile. When two isolates provided a similar profile the *gmhA* amplicons were re-digested and visualised on an agarose gel next to each other. This either confirmed identity or if a variation was present. Table 4 summarises the isolates examined in this study.

Figure 10 illustrates physical maps of *C. jejuni* and *C. coli* genomes amplified by primers 96.01 and 94.293.

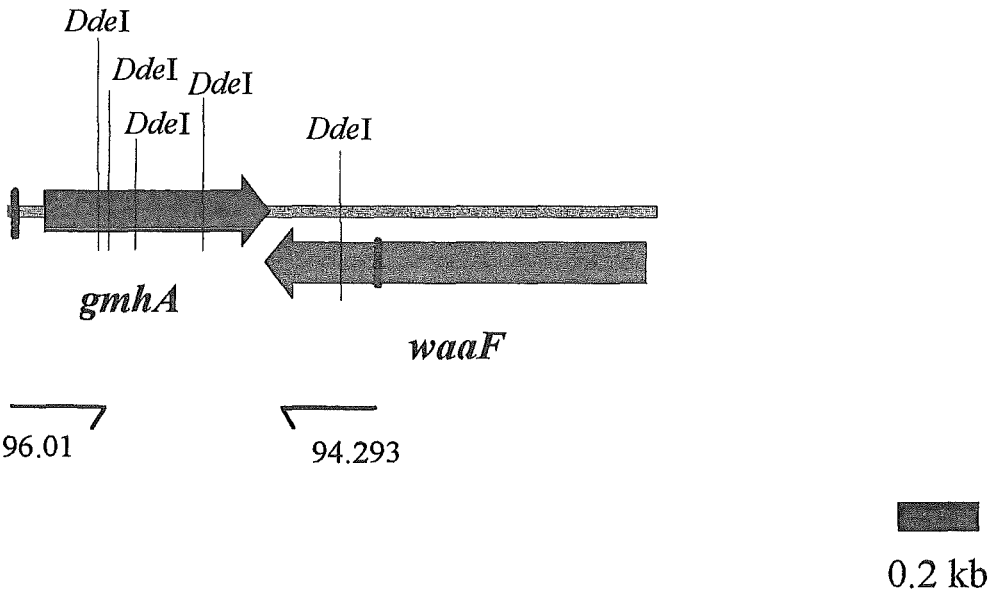


Figure 10a: Physical map of the *gmhA* gene in C. *jejuni* isolate NCTC 11168. The 900 bp PCR amplicon generated using primers 96.01 and 94.293 is shown including *Dde*I digestion sites located throughout the amplicon.

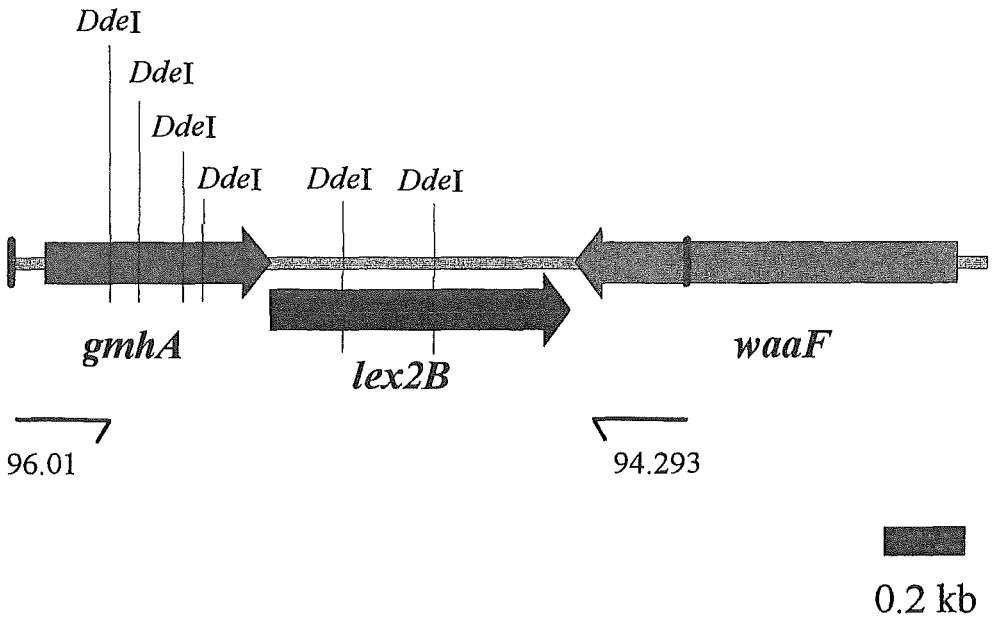


Figure 10b: Physical map of the *gmhA* gene in *C. coli* isolate M275. The 1.6 kb PCR amplicon generated using primers 96.01 and 94.293 is shown, including *Dde*I digestion sites located throughout the amplicon.

Assigning *gmhA* profiles was done by awarding the first profile observed the designation 1 and each new profile labeled with the next consecutive number. The RFLP procedure provided a further 6 *gmhA* profiles (7 including the previously mentioned *gmhA* 7). Figure 11 illustrates the different profiles observed in this study.

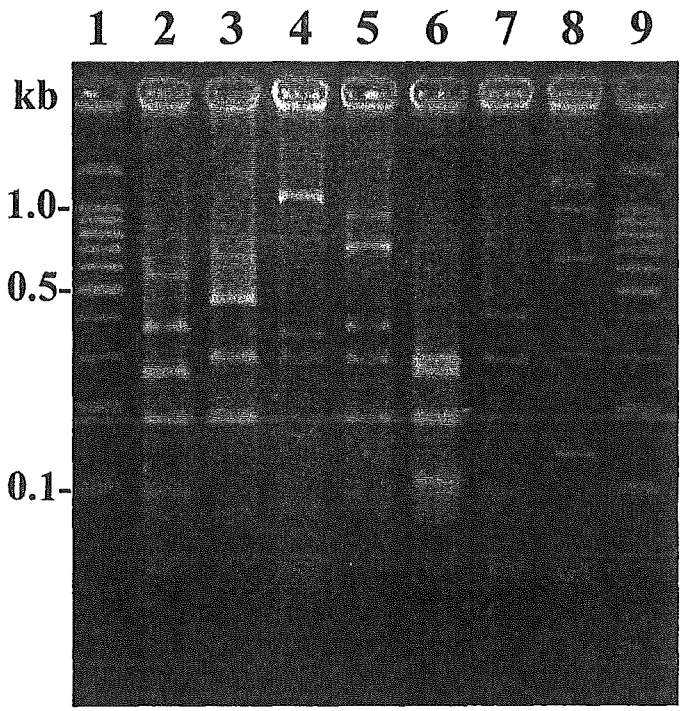


Figure 11: Agarose gel photo illustrating the different *gmhA* profiles observed in this study. Lanes 1 and 9, 100 bp DNA ladder (Promega); Lane 2, *gmhA* 1 NCTC 11168; Lane 3, *gmhA* 2 ZF320J; Lane 4, *gmhA* 3 generated by a 1.6 kb amplicon MB617S; Lane 5, *gmhA* 4 LS897W; Lane 6, *gmhA* 5 FD131W; Lane 7, *gmhA* 6 EU369T; Lane 8, PCR amplified multiple band profile designated *gmhA* 7 BB193H.

Isolates were unequally distributed into different *gmhA* types. Figure 12 shows the distribution of isolates into *gmhA* profiles. *gmhA* 1, which included NCTC 11168, was the largest group observed with 30 isolates (28.6 % of the original sample). Of the isolates that generated a PCR-RFLP profile, *gmhA* 5 (8.6 % of the original sample) was the second largest group in this study. However the largest equal group included those isolates that did not amplify under PCR condition (27.6 %), with 7.6 % of the isolates not providing a RFLP profile. These isolates may not have generated the RFLP profile as the original PCR amplicon was too faint or the profile was too faint or not present on the agarose gel to allow accurate classification into a *gmhA* category.

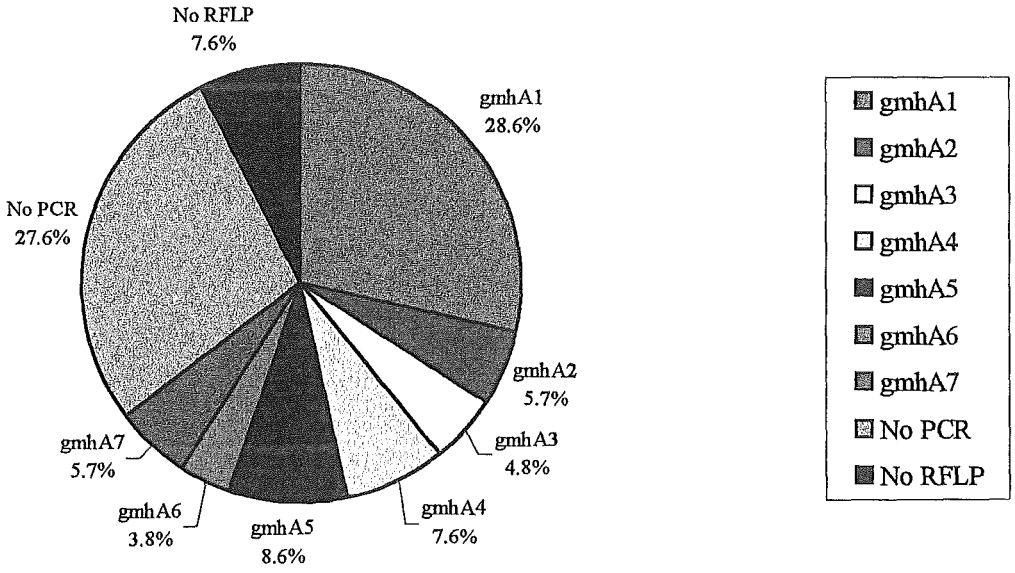


Figure 12: Distribution of the clinical isolates into *gmhA* groups.

Dendrograms were generated as per *flaA*. Figure 13 shows the relationship of the five *gmhA* classifications based on a 900 bp PCR-RFLP profile. *gmhA* 3 and 7 were excluded from this dendrogram as the PCR amplicons generated were not directly comparable to the 900 bp amplicon. Figure 13 shows two major classes of the *Campylobacter gmhA* gene. The dendrogram shows *gmhA* 1 and 5 to be related with 60 % similarity. These related *gmhA* types probably share a common ancestral *gmhA* gene. It is difficult to assess whether *gmhA* 7 shares a common *gmhA* gene to the remaining isolates, as this group repeatedly provided the multiple band profile, suggesting a possible divergence in the *gmhA* gene. The *gmhA* 3 amplicon, containing a third gene is anticipated to have significant differences within the *gmhA* gene (section 4.3).

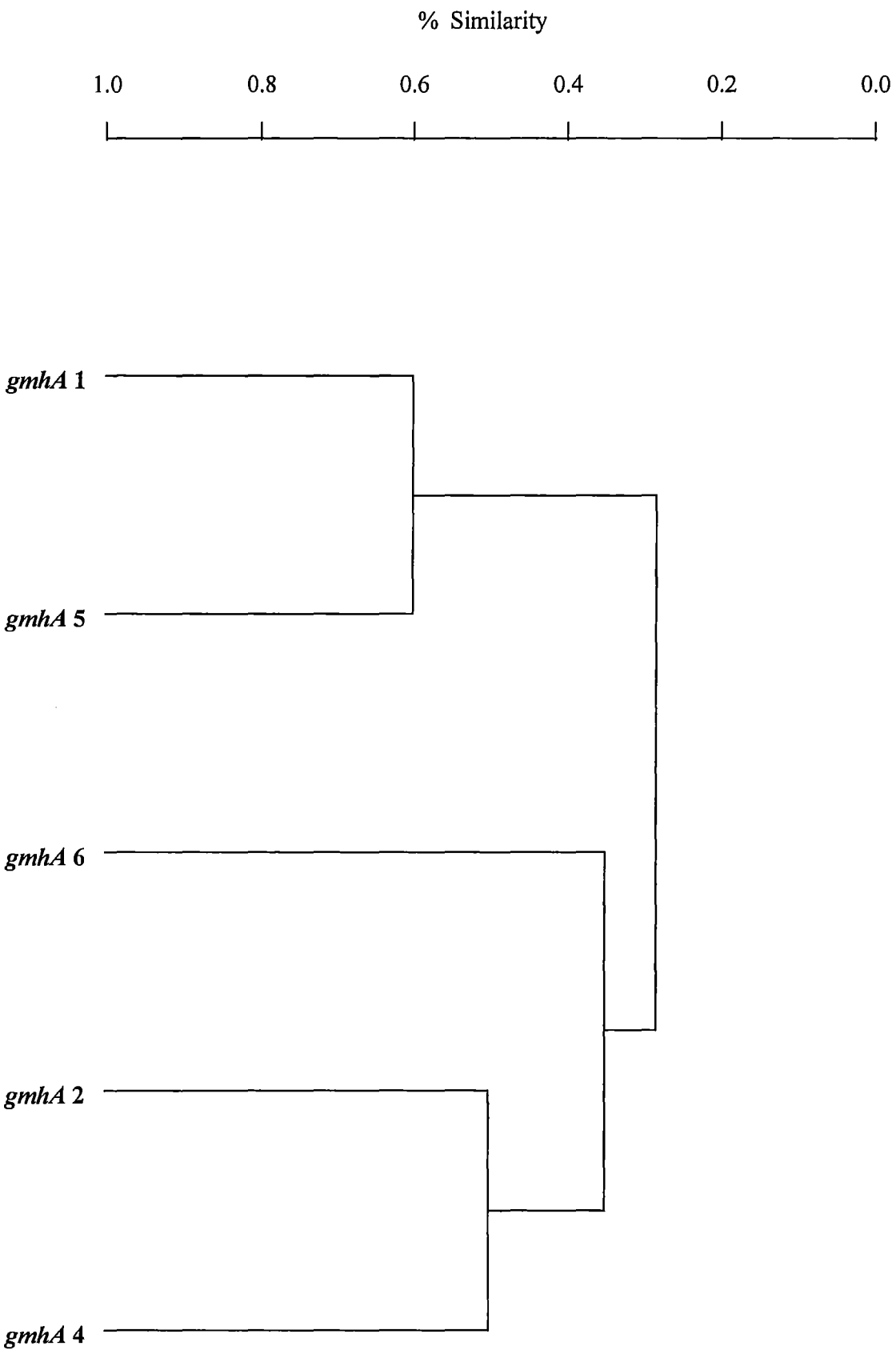


Figure 13: Dendrogram illustrating the relatedness of the clinical isolates using the *gmhA* gene.

From these experiments it was possible to determine the degree of discrimination the *gmhA* gene provided and whether it is useful as an epidemiological marker. Using Simpson's index of diversity, the isolates that provided an RFLP profile (n=66) generated a D value of 74 %. This meant that when examining a new isolate, the isolate could be classified into a pre-existing *gmhA* type in 74 out of 100 cases. While this is not as satisfactory as *flaA* typing it may still be a valuable marker used in conjunction with *flaA* (section 3.4). However when calculating the D value based on the sample size of 105 isolates, 90 % of the time a new isolate should be able to be classified into an existing category. Again, as with *flaA*, a large portion of this sample was unable to provide a PCR amplicon therefore this value may be somewhat misleading.

3.4 CLINICAL ISOLATES: COMBINED *flaA* AND *gmhA* PCR-RFLP ANALYSIS.

This section analyses only the isolates that generated both a *flaA* and *gmhA* PCR amplicon. From the 105 clinical isolates 70 (66.7 %) generated both a *flaA* and *gmhA* PCR amplicon. Included in this number was the control isolate NCTC 11168. From these 70 isolates 62 (59 % of the original sample) generated detectable RFLP profiles.

The aim of this section of the thesis was to determine if the application of a second marker, *gmhA*, an unlinked gene on the *Campylobacter* genome, was able to further discriminate between isolates classified by *flaA*. It was observed that a higher level of discrimination did occur when *gmhA* was included in the analysis. For example *flaA* group 2 contains 11 isolates. Combining the *gmhA* allele with *flaA* typing, *flaA* 2 was further divided into four subgroups (*flaA* 2/*gmhA* 1 (n=1), *flaA* 2/*gmhA* 2 (n=5), *flaA* 2/*gmhA* 5 (n=2), and *flaA* 2/*gmhA* 7 (n=3)). Clearly an additional marker increased the level of discrimination.

Increased discrimination was observed for a number of *flaA* groups. *flaA* 4 contained four isolates, these separated into 3 subgroups, *gmhA* 3 (n=1), *gmhA* 6 (n=1) and *gmhA* 7 (n=2). The largest group, *flaA* 6 (n=15), could be separated into two additional subgroups using *gmhA*, *gmhA* 1 (n=14) and *gmhA* 4 (n=1). Figure 14 is a dendrogram illustrating the relationship between isolates combining both gene markers.

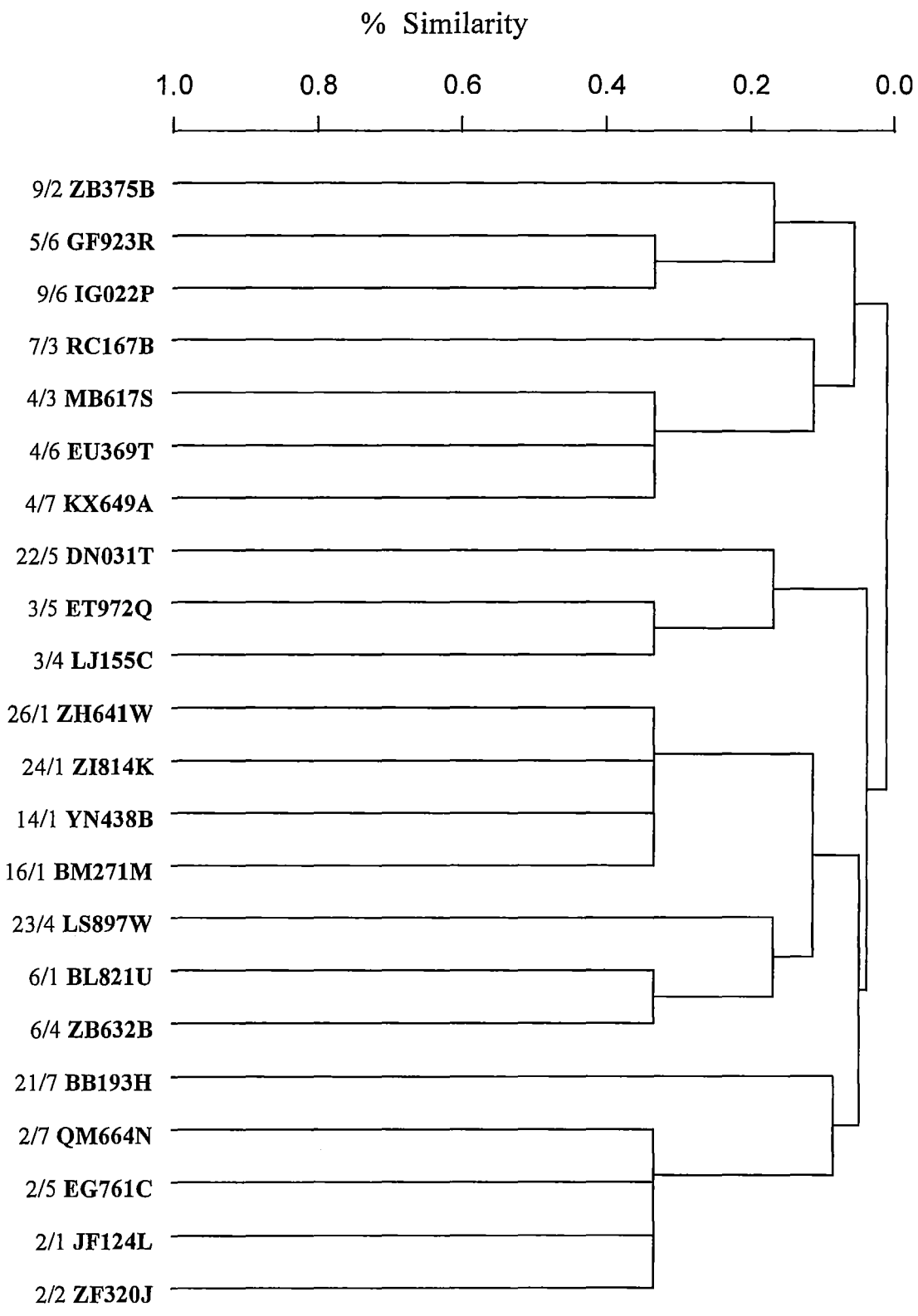


Figure 14: Dendrogram illustrating the discrimination of isolates using a combined analysis of *flaA* and *gmhA* markers. The first number to the left of the isolate name indicates the *flaA* classification, whereas the second number indicates the *gmhA* classification.

The dendrogram illustrates two major clusters of isolates that are relatively unrelated to one another. Isolates ZB375B, GF923R, IG022P, RC167B, MB617S, EU369T and KX649A are in one cluster. The remaining isolates group into the second larger cluster. This may suggest different evolutionary histories.

flaA 22 consisted of three isolates that were obtained from the same hospitalised patient. It is not surprising that these 3 isolates typed as the same *flaA* and *gmhA* type. This group could not be discriminated further on the basis of these two markers, providing an internal control for this method. From the data presented here, it appears that one strain was responsible for the infection of this patient, not multiple strains. It was often observed that multiple isolates from the same patient were indistinguishable. This is not to say that another marker could not discriminate between these isolates further, but based on the information obtained in this study, they appear indistinguishable.

Several *flaA* groups were only represented by one *gmhA* type and therefore addition of this marker to the analysis did not increase overall strain resolution. This may be due to the fact that the *flaA* group only comprised of one isolate or that some isolates only generated a *flaA* profile but not a *gmhA* profile. The former case is illustrated by *flaA* 24, which only comprised of one isolate, ZI814K. The latter case is illustrated by *flaA* 16. In *flaA* 16 (n=10), nine isolates provided *gmhA* 1 profile, while the other isolate gave no *gmhA* result.

The level of discrimination achieved by these two genes when combined was calculated using Simpson's index of diversity. Using a sample size of 62 isolates that had provided both *flaA* and *gmhA* RFLP profiles a D value of 92 % was calculated. This means that a new isolate will be classified into the pre-existing profiles 92 % of the time. This is encouraging in that the use of these two markers provide a relatively quick and reproducible scheme. However, increased sensitivity would be desirable.

3.5 MULTILOCUS ENZYME ELECTROPHORESIS (MLEE).

MLEE procedures were applied to the largest *flaA* group identified in this study. This group, *flaA* 6, contained 15 isolates. Application of *gmhA* had not discriminated this

group much further (see below). Therefore MLEE analysis, detecting the presence of specific enzymes in an organism, was applied to determine if the isolates were, in fact, identical or heterogeneous.

flaA 6 consisted of 15 isolates including NCTC 11168. g*mhA* typing further classified this cluster into two subgroups, *gmhA* 1 (n=14) and *gmhA* 4 (n=1). Two enzymes, malate dehydrogenase NAD^+ (MDH) and malate dehydrogenase $NADP^+$ (ME) (appendix 3), were used to determine strain differences by MLEE. While two other studies have reported success with at least nine enzymes (Patton *et al* 1991, Aeschbacher and Piffaretti 1989), due to time constraints only two enzymes were used. Two other enzymes, catalase and phe-leu peptidase, were trialled without adequate results.

For MLEE to provide satisfactory results, cell numbers of each isolate should be approximately $2x10^8$ cfu/ml. Cell numbers varied in this experiment from $1x10^7$ cfu/ml to $6.7x10^8$ cfu/ml; table 5 shows the isolate and corresponding cell numbers. When examining the 15 isolates with ME, 13 isolates provided a positive result for this enzyme, generating two different alleles. However MB321A and RC722O returned a null result. These were recorded as a null to indicate the possible lack of enzyme in these isolates. It was possible that RC722O may not have had an adequate cell number to generate a result. MB321A extracts contained an acceptable number of cells but still did not generate a result. Figure 15 shows cellulose acetate plates stained with ME.

When isolates provided an identical allele profile to one previously observed, the isolates were analysed repeatedly to determine if they were identical. From these observations (data not shown) it was determined that the isolates recorded as allele 2 were in fact identical.

Table 5: Cell numbers of the isolates used in this study.

Isolate Name	No. of Cells
NCTC 11168	$1.3x10^8$
BL821U	$7x10^7$
CE934Q	$1.9x10^8$
CA755I	$3x10^8$
ZB632B	$6x10^7$
R923	$3x10^7$
S052	$6.7x10^8$
LT038S	$2.9x10^8$
LS466P	$5x10^7$
MB200L	$1.1x10^8$
MB321A	$5.5x10^8$
QP543U	$5x10^7$
RE097T	$6.2x10^8$
RF185D	$2x10^8$
RC722O	$1x10^7$

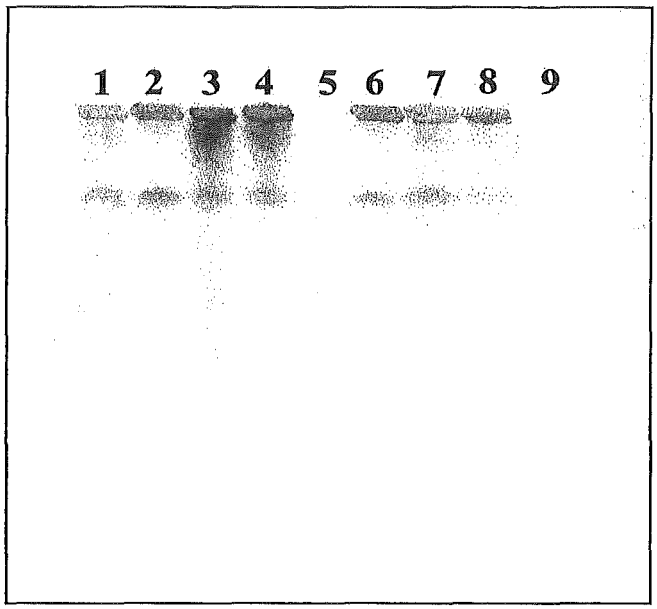


Figure 15a: *flaA* group 6 isolates visualised on cellulose acetate plates stained with ME. Lane 1, NCTC 11168; Lane 2, R923; Lane 3, S052; Lane 4, CA755I; Lane 5, marker; Lane 6, BL821U; Lane 7, CE934Q; Lane 8, MB200L; Lane 9, RC7220.

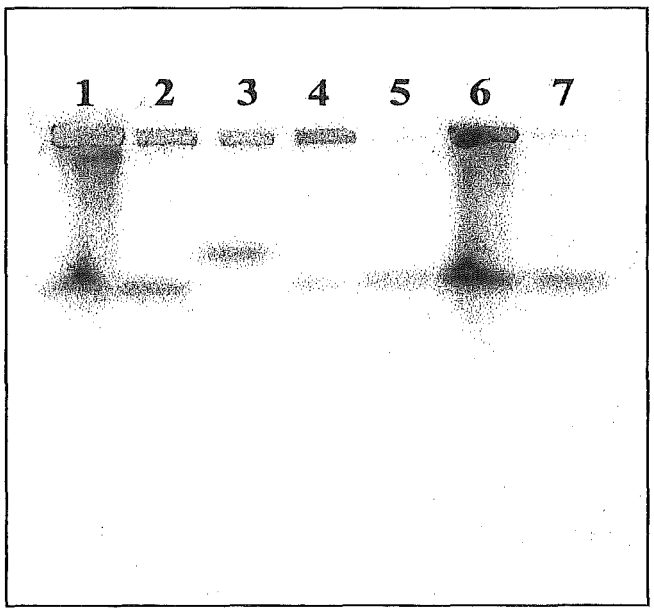


Figure 15b: *flaA* group 6 isolates visualised on cellulose acetate plates stained with ME. Lane 1, RE097T; Lane 2, RF185D; Lane 3, ZB632B; Lane 4, MB321A; Lane 5, QP543U; Lane 6, LT038S; Lane 7, LS466P.

MDH isozyme analysis revealed that 11 isolates had an enzyme with identical migration properties. Three different profiles, representing four alleles, were generated with MDH. One isolate (ZB632B) contained a second allele and a third isolate (RC722O) had two alleles. Two isolates, MB321A and QP543U, lacked an allele. The isolates that did not generate an allele with this enzyme should have enough cells present, although MB321A was negative in both trials. QP543U had generated an allele with the previous enzyme, therefore the results of MDH indicate a lack of a functional enzyme present in this isolate. It may be important to increase the cell numbers of these isolates to determine whether cell numbers played a role in the lack of allele generated.

MB321A a faecal isolate, did not provide results via MLEE, however MB200L, isolated from the blood of the same patient was positive in both instances. Further experiments, possibly incorporating other enzymes will need to be attempted to determine if an error did occur or if these two isolates are different. Figure 16 show cellulose acetate plates stained with MDH.

Isolates that generated an allele identical to one previously observed were analysed again to determine identity (data not shown). This procedure allowed accurate classification of isolates between cellulose acetate plates.

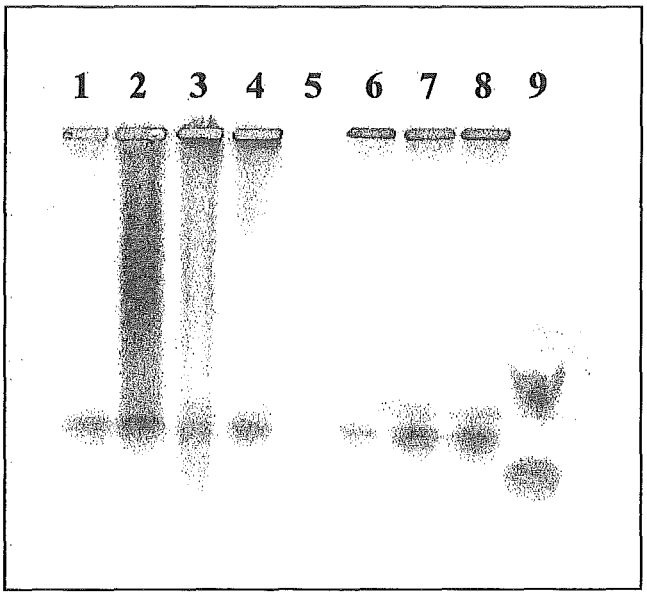


Figure 16a: *flaA* group 6 isolates visualised on cellulose acetate plates stained with MDH. Lane 1, NCTC 11168; Lane 2, R923; Lane 3, S052; Lane 4, CA755I; Lane 5, marker; Lane 6, BL821U; Lane 7, CA934Q; Lane 8, MB200L; Lane 9, RC7220.

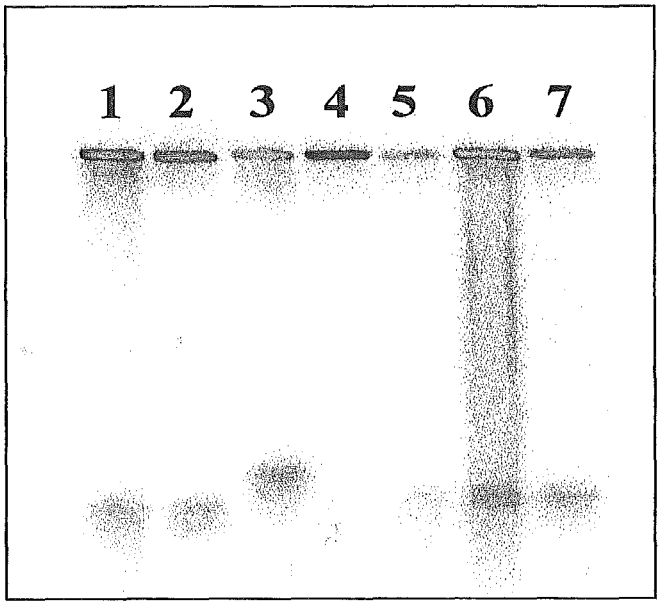


Figure 16b: *flaA* group 6 isolates visualised on cellulose acetate plates stained with MDH. Lane 1, RE097T; Lane 2, RF185D; Lane 3, ZB632B; Lane 4, MB321A; Lane 5, QP543U; Lane 6, LT038S; Lane 7, LS466P.

Using MLEE the 14 *flaA* 6 isolates could be discriminated into 5 subgroups. Figure 17 is a dendrogram illustrating the relatedness of these isolates. The x-axis indicates percent genetic similarity. It is not surprising that isolate ZB632B is unlinked to the other isolates (as shown by the dendrogram). This was the sole isolate that was in *flaA* 6 with a unique *gmhA* classification. Three groups were represented by one isolate. QP543U only produced a band for ME therefore being 60 % related to the remainder of isolates. RC722O, as mentioned previously, did not appear to have an ME allele and gave a unique MDH profile in this sample, therefore being 70 % unrelated to the remainder of the *flaA* 6, *gmhA* 1 group. These results show that while ZB632B is related by *flaA*, the majority of evidence suggests that this isolate is significantly different from the remaining isolates in this group. Further the majority of strains (*flaA* 6, *gmhA* 1) appear to be identical, therefore suggesting a clonal link between isolates. However more enzymes should be assessed to make any strong conclusions.

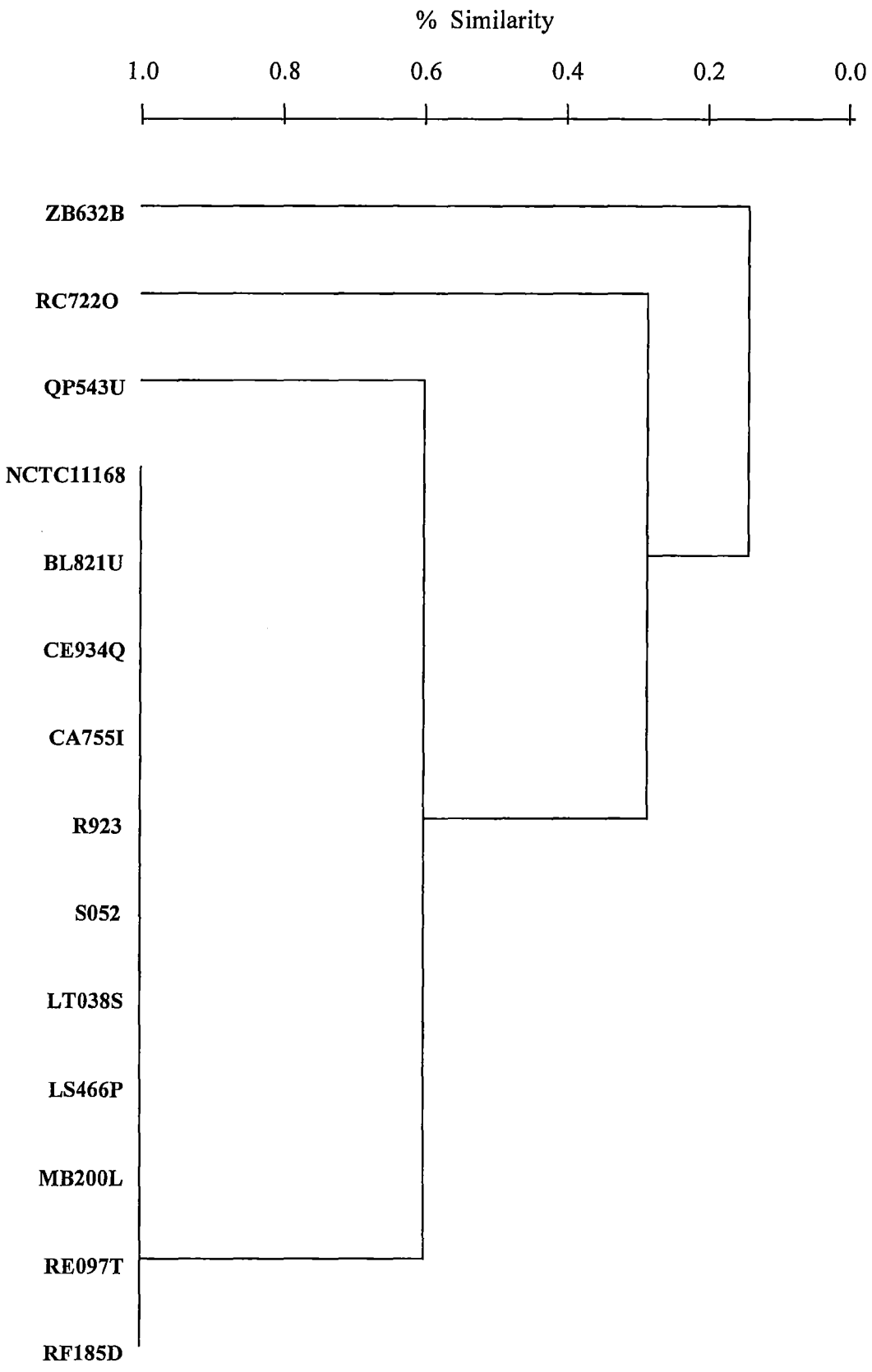


Figure 17: Dendrogram of *flaA* group 6 analysed by MLEE.

3.6 ENVIRONMENTAL (SHEEP) ISOLATES.

3.6.1 ENVIRONMENTAL ISOLATES.

C. jejuni isolates (n=20) were obtained from Stan Fenwick and Fasi Ahmed (Massey University). These isolates were cultured from sheep and were identified as *C. jejuni* by a range of tests, including hippurate hydrolysis at Massey University. Isolates were retested for hippurate hydrolysis at Canterbury University and two isolates, CJS-71 and CJS-81, returned a negative result. These isolates may in fact be *C. coli* or another *Campylobacter* spp..

3.6.2 *flaA* TYPING.

Concurrent to studying the human clinical isolates, 20 *C. jejuni* isolates from sheep were examined using PCR-RFLP of the *flaA* gene. Procedures were identical to those used for the clinical isolates. Of the 20 isolates, 18 (90 %) generated a 1.7 kb amplicon. NCTC 11168 served as a control throughout the experiments. Hippurate hydrolysis of the two *flaA* PCR-negative isolates (CJS-71, 81) had previously suggested that these isolates were not *C. jejuni*, but possibly another *Campylobacter* spp.. Time did not permit further analysis of these samples.

The remaining 18 *flaA* amplicons were restriction digested using *Dde*I to generate a RFLP profile. 16 isolates (80 % of the original sample) generated a profile. The two isolates that did not generate a RFLP profile were too faint to accurately classify into a category, this may be due to the PCR amplicon being too faint or not enough template used in the digestion. Table 6 summarises the respective *flaA* profiles obtained in this study. Four *Dde*I-RFLP profiles were generated from the *flaA*-PCR amplicons from sheep. All four of these profiles were observed in the human cases described above. The similarity of *flaA* types in the environmental samples to the clinical setting suggests that there maybe a limited number of *flaA* profiles possible in nature. *flaA* 6 was the largest group, containing five isolates (25 % of the original sample). *flaA* 16 also contained five isolates. Figure 18 shows the distribution of the *flaA* profiles. Due to the small sample size a dendrogram was not generated. The D value was calculated combining the clinical isolates with the environmental isolates. This provided a D value of 94 % when a sample size of 125 was used in the calculation.

Table 6: Summary of environmental isolates used in this study and respective *flaA* and *gmhA* classifications.

Isolate Name	Source	*flaA* type	*gmhA* type
CJS-72	Sheep	6	1
CJS-75	Sheep	6	1
CJS-74	Sheep	6	F
CJS-84	Sheep	6	NB
CJS-85	Sheep	6	NB
CJS-78	Sheep	14	1
CJS-79	Sheep	14	1
CJS-83	Sheep	14	1
CJS-73	Sheep	16	1
CJS-77	Sheep	16	1
CJS-82	Sheep	16	1
CJS-86	Sheep	16	1
CJS-87	Sheep	16	1
CJS-88	Sheep	21	1
CJS-90	Sheep	21	1
CJS-89	Sheep	21	3
CJS-76	Sheep	F	1
CJS-80	Sheep	F	5
CJS-81	Sheep	NB	1
CJS-71	Sheep	NB	NB

CJS = *Campylobacter jejuni* sheep.
F = Faint RFLP pattern, not distinguishable.
NB = No PCR Band.

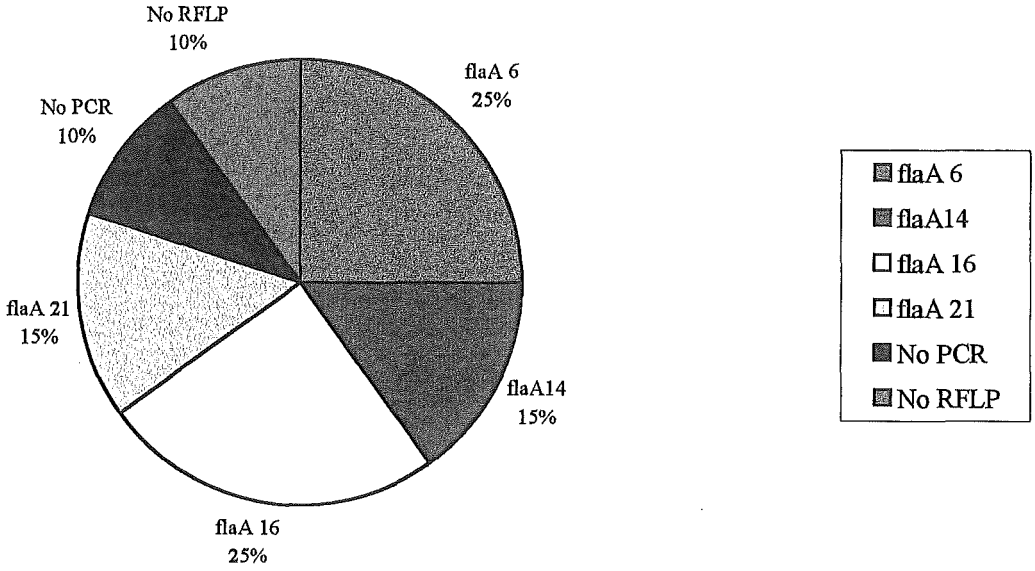


Figure 18: Distribution of environmental isolates into *flaA* classifications.

3.6.3 *gmhA* TYPING.

Of the 20 isolates three did not generate a PCR amplicon using *gmhA*. This included one isolate (CJS-71) that had not generated an amplicon under *flaA*, therefore this isolate may be *C. coli* or another thermophilic *Campylobacter*. Whereas CJS-81 generated a 900 bp amplicon with *gmhA*. One isolate CJS-89 provided a 1.6 kb amplicon suggesting the possibility of a third gene as seen in clinical isolates YP502F, LG430I, MB617S, ZP028D and RC167B. The remaining isolates generated the 900 bp amplicon previously observed in the clinical experiments.

17 *gmhA* amplicons (85 % of the original sample) were restriction digested using *Dde*I and an RFLP profile was generated for 16 (80 %) of the isolates. CJS-74 was not classified under *Dde*I-RFLP analysis as the profile generated was too faint to accurately assign to a category. *Dde*I-RFLP analysis of CJS-89 generated a *gmhA* 3 profile, this had been observed in the clinical isolates that had generated the 1.6 kb amplicon. Table 6 summarises the isolates used in this study and their respective *gmhA* classification.

Three *gmhA* profiles were observed from the environmental isolates, these were *gmhA* 1, *gmhA* 3 and *gmhA* 5. These profiles had previously been observed in the clinical isolates, therefore suggesting no variation in *gmhA* alleles at least on a gross restriction enzyme scale between sheep and those found in humans. As observed in the clinical experiments *gmhA* 1 contained the largest number of isolates, with 14 (70 % of the original sample). Only 1 isolate (5 %) fell into the *gmhA* 5 category. Figure 19 shows the distribution of the *gmhA* profiles amongst this group. A dendrogram was not generated as the profiles observed in the environmental isolates were previously observed in the clinical isolates, therefore no additional information would have been provided by the dendrogram. Combining the environmental samples with the clinical isolates, a D value was calculated. With a sample size of 125 isolates, 86 % of the time a new isolate will be able to be classified into an existing category using *gmhA* as a marker. This is not as satisfactory as *flaA*.

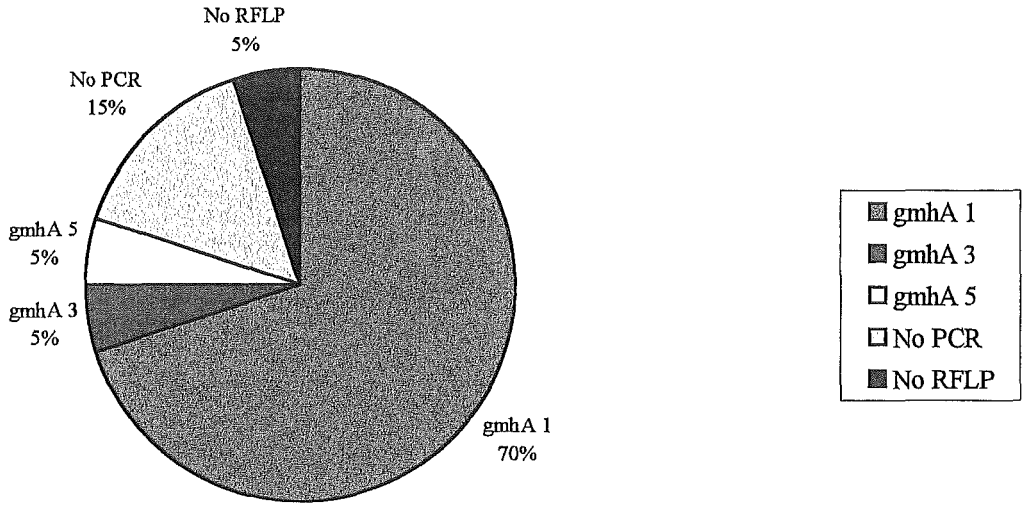


Figure 19: Distribution of environmental isolates into *gmhA* classifications.

3.6.4 COMBINED *flaA* AND *gmhA* PCR-RFLP.

Of the 20 *C. jejuni* sheep isolates, 16 (80 %) provided a PCR amplicon for both *flaA* and *gmhA*. Of these 16 isolates, 13 (65 % of the original sample) had generated an RFLP profile. Three isolates (CJS-74, 76 and 80) generated faint RFLP profiles for either *flaA* or *gmhA*, therefore these isolates could not be accurately characterised. The ability to discriminate the environmental isolates further with *flaA* and *gmhA* combined was not as satisfactory as in the clinical isolates. Of the 13 RFLP positive isolates, there were four *flaA* groups, with 12 isolates represented by the same *gmhA* classification, *gmhA* 1. The remaining isolate, CJS-89, was classified as *gmhA* 3.

CHAPTER IV

DISCUSSION

4.1 ISOLATE INFORMATION.

Campylobacteriosis caused by *C. jejuni* is one of the most common causes of bacterial gastroenteritis worldwide and has been a notifiable disease in New Zealand since 1980. The number of notified cases in New Zealand are increasing on a yearly basis. Campylobacteriosis is reported to be higher in males than females and primarily affects the very young (1-4 year olds) and young adults (20-29 year olds) (Allos and Blaser 1995, Butzler and Skirrow 1979, Blaser and Reller 1981). At an estimated cost of $582,194 for the Canterbury region in 1995 and $4.48 million nationally this organism is of great interest to public health (Withington and Chambers 1997).

The number of isolates collected per month in this study ranged from 1 to 23, with an average of 9.5 cases per month. During this study December 1997 (n=23) recorded the highest number of cases at Christchurch Public Hospital. In January 1998 (n=12) the number dropped, before increasing again in February 1998 (n=18). However, it was observed that the number of cases reported to the Southern Regional Health Authority increased in December 1997 (n=130), with January 1998 reporting the largest number of cases (n=195), before decreasing again in February 1998 (n=160).

The trends reported in the literature (Brieseman 1994) regarding sex disposition and age were in general observed in this study. It was found however, that the second most prevalent category in this study was 30-39 year olds, consisting of eight males and eight females. A higher number of cases may be reported from this age group for several reasons; they maybe more likely to seek medical attention in order to continue employment or be more severely affected. The 1-4 year age group may also be under-represented for numerous reasons. Infants may experience general diarrhetic illness at a higher frequency than other age groups, so parents may be more willing to take "a wait and see approach" to the disease outcome.

Cases of campylobacteriosis in this study were analysed for a temporal and geographical link. In one instance, three cases from Ashburton, involving two males (ZN474U and ZN807W) and one female (ZN244B) of approximately the same age, were collected within one week of each other. While no epidemiological data regarding source of infection was reported, this study observed that the two males had an identical strain of *C. jejuni*. The isolate collected from the female did not generate a result, therefore it can not be concluded to be identical to that of the males. It was also observed that in December 1997, two cases of campylobacteriosis were reported from 26 year old males from Kaiapoi. Both cases generated the same *flaA* and *gmhA* RFLP profiles, strongly suggesting the same strain was responsible for both illnesses.

Cases of campylobacteriosis are often sporadic in nature, however it is possible that under-reported outbreaks may occur. Stehr-Green *et al* (1991) investigated two reported cases of campylobacteriosis in Canterbury during 1990. Both of these cases were linked to a camp and convention center in Canterbury. Due to epidemiological and microbiological investigations, a further 42 cases were identified. This investigation illustrates the potential occurrence of under-reported outbreaks. During the present study the three previously mentioned cases from Ashburton may be an example of an unreported outbreak, of which more cases were not notified.

In the present study 15 patients provided more than one isolate during the course of their illness. When examined by *flaA* and *gmhA* PCR-RFLP analyses, each isolate that generated a profile was shown to be identical to isolates from the same patient. This suggests that only one strain was responsible for these infections. While the sample size is clearly limited, *flaA*/*gmhA* PCR methods should be able to provide insight into the ecology of *C. jejuni* in a human host.

4.2 CLINICAL TYPING USING *flaA*.

Clinical differentiation of *C. jejuni* and *C. coli* strains has been based solely on the ability of *C. jejuni* to hydrolyse hippurate, whereas *C. coli* does not. This test is somewhat subjective as hippurate negative *C. jejuni* strains are occasionally observed (Totten *et al* 1976). *Campylobacter* spp. are relatively inert with regard to routine

biochemical tests used for species identification. The detection and identification of these organisms by PCR is now being recognised as a reliable alternative to the traditional tests (Harmon *et al* 1997).

A suitable typing system for bacterial isolates needs to be robust, highly reproducible and have a high discriminatory power. Fischer and Nachamkin (1991) observed differences in the nucleotide structure of the *flaA* gene in *C. jejuni*, indicating that this gene could be exploited as an epidemiological marker. Nachamkin developed a typing system based on PCR-RFLP analysis of the flagellin gene, *flaA*, observed in *C. jejuni* and *C. coli*. The amplicon generated by this combination of *flaA* primers was approximately 1.7 kb; when this amplicon was examined using the restriction enzyme *Dde*I in RFLP analysis, distinctive profiles were generated. Comparison of the *Dde*I generated RFLP profiles forms the basis of Nachamkin's system. *flaA* gene analysis was found to be highly reproducible in the isolates that generated results, robust and provided a high level of discrimination in the present study.

flaA-RFLP profiling is increasingly being used as a typing method for epidemiological studies (Nachamkin *et al* 1996, Owen *et al* 1994, Meinersmann *et al* 1997). Most studies note the usefulness and high level of discrimination that PCR-RFLP analysis of the *flaA* gene provides. On the basis of these observations this technique was chosen for the present study. *flaA* typing had previously been applied to *Campylobacter* isolates obtained from hospitalised patients at Princess Margaret Hospital, Christchurch (Hasse *et al* 1995), and sporadic *C. jejuni* cases from the human environment (Armstrong 1997). This method provided a useful means to determine the extent and classification of *flaA* groups in the Christchurch Public Hospital environment during this study.

83.8 % of 105 isolates from 84 patients generated the expected 1.7 kb *flaA* amplicon. As noted previously (Comi *et* al 1996, Fischer and Nachamkin 1991) it is not possible to separate *C. jejuni* and *C. coli* by *flaA*. These observations were supported in the present study. *C. coli* isolates RC167B typed as *flaA* 7, while QY139W and RD177L typed as *flaA* 9. As both of these profiles were observed in isolates of *C. jejuni*, it was not possible to distinguish between species. In this study isolates that tested hippurate positive were considered to be *C. jejuni*. Use of a second genetic test, possibly

amplification of species specific regions of the 23S rRNA could be used for confirmation of the *flaA* hippurate positive isolates as *C. jejuni*.

Nachamkin *et al* (1996) found that 85 % of the 404 isolates tested were amplified by *flaA*-PCR using chromosomal templates extracted by boiling whole cells. This procedure is simple to perform, but is dependent on inoculum size, as too much inoculum (>50,000 cells) can inhibit the PCR reaction. PCR procedures in the present study involved the lysis of bacteria in a Corbett thermal sequencer before the addition of *Taq* polymerase and PCR reaction buffer. It is possible that the amount of cells used for PCR could have inhibited the PCR reaction. This remains a possibility for the 17 isolates not generating the expected amplicon.

The classification of RFLP profiles in this study was not as previously described by Nachamkin. Due to the expense and unavailability of the ProRFLP image analysis software, the results of this study were unable to be directly compared to Nachamkin. Nachamkin *et al* (1996) described 83 *flaA* types based on 404 *C. jejuni* and *C. coli* isolates. It is possible that the current 17 types observed in this study fall into this existing system although some isolates may represent a unique *flaA* type. It may be useful for future reference to compare the database of *flaA* types generated by this study with those described by Nachamkin. Eleven of the *flaA* types identified had previously been observed by Armstrong (1997), however six were new to this study.

The *flaA* types identified in this study illustrate that there is not one particular *C. jejuni* or *C. coli* strain responsible for human and animal infection. From these results it appears a number of *flaA* types of *C. jejuni* and *C. coli* are responsible for infection. However it was observed that some strains (e.g., *flaA* 6) were isolated at a greater frequency from humans and animals than others during this study. Armstrong (1997) observed *flaA* 1 (n=7 from a sample size of 41) to be the most common type identified from isolates obtained from human faeces at MedLab South Ltd, Canterbury, during the summers of 1995/96 and 1996/97. *flaA* 6 (n=2) was equal to *flaA* groups 3, 4 and 5, therefore not supporting the trend of the present study, although low sample sizes were involved.

NCTC 11168 was typed in the present study as *flaA* 6 and this strain is classified as HS:2 according to Penner serotyping. In Nachamkin's typing system NCTC 11168 is classified as *flaA* 7 (Nachamkin *et al* 1996). While NCTC 11168 is HS:2, it is not known whether the remaining isolates in this group are also HS:2. In Nachamkin's study *flaA* 7 also contained isolates typed as HS:1, HS:10 and HS:17, therefore it appears that this group is relatively heterogeneous. Those isolates typed as *flaA* 6 in the present study may classify into different HS serotypes.

Dendrogram analysis suggests that closely related isolates have derived from a common ancestor. *flaA* 3, 21, 22 and 26, which are not closely related to the main cluster (over 70 % unrelated), appear to have deviated from a common ancestor some time ago. Wassenaar *et al* (1995) observed that *C. jejuni* has substantial genetic diversity within the flagellar region. They demonstrated recombination within the genome, as well as recombination after the uptake of exogenous DNA. *flaA* and *flaB* are highly similar, therefore subject to intergenic recombination. This recombination may explain the presence of 17 different *flaA* types in this study. Due to horizontal gene transfer or intergenic recombination, assessment of clonality of isolates may be difficult (section 4.7).

4.3 CLINICAL TYPING USING *gmhA*.

LPS is used as the basis of the heat stable (HS) serotyping scheme of *C. jejuni*, identifying up to 60 serotypes (Aspinall *et al* 1993a, Moran *et al* 1991). LPS molecules contain considerable serological heterogeneity between isolates (Beer *et al* 1986, Perez-Perez *et al* 1985). The *gmhA* gene identified in *C. jejuni* and *C. coli* by Upritchard (1997) was observed to have heterogeneity between two strains examined. On the basis of this research, the *gmhA* gene was chosen to act as a second marker in the present study. The recently submitted whole genome sequence of NCTC 11168 (Sanger) clearly indicates that *gmhA* is unlinked to the *flaA* gene, therefore any result generated by this second marker, *gmhA*, should discriminate *flaA* further.

Of the 105 clinical isolates examined using primers specific for amplification of the *gmhA* gene and a portion of the *waaF* gene, 71.4 % of the sample generated either a

900 bp, 1.6 kb fragment or a multiple band profile. There were five isolates that generated a 1.6 kb amplicon. One isolate, M275, has recently been described as *C. coli*. During the course of sequencing and analysis of *waaF* from M275 a second gene, which had sequence identical to the *lex*2B gene of *H. influenzae* (Jarosik and Hansen 1994) was observed. Upritchard (1997) also showed that *gmhA* was linked to *lex*2B. This gene was not present in all isolates examined in this study. Work in the laboratory is currently under way to characterise this gene, however this is out of the scope of the present study. The majority of isolates examined generated the anticipated 900 bp fragment, indicating a lack of the *lex*2B gene.

Isolates that generated a 1.6 kb amplicon under PCR conditions were restriction digested with *Dde*I and classified as *gmhA* 3. This classification was observed in both *C. jejuni* and *C. coli*, therefore suggesting it was not a characteristic of either species. On the basis of the presence of a 1.6 kb amplicon, these isolates were assumed to contain the *lex*2B gene.

Six of the isolates examined generated a multiple band profile (designated *gmhA* 7) under PCR conditions. These isolates were examined a second time generating the same result. These isolates were not subjected to RFLP analysis as interpretation of the profile would have been meaningless. When comparing RFLP profiles it is important that amplicons of the same size are compared. With *gmhA* 7 generating a multiple band profile, RFLP analysis would not have allowed accurate and equal comparison with those of 900 bp.

Isolates with a clean 900 bp amplicon were restriction digested with *Dde*I. Analysis of the 900 bp isolates generated a further 5 PCR-RFLP profiles. As the present study was the first to use *gmhA* as a marker, no previous classifications for RFLP profiles existed. It was therefore decided to assign each profile in the order they were observed.

gmhA has been observed in *E. coli, Haemophilus, Helicobacter, Neisseria* and other Gram-negative prokaryotes. The *gmhA* gene is likely to be present in all *C. jejuni* and *C. coli* isolates, including those classified as *gmhA* 7 in this study. *gmhA* has been identified to encode for the enzyme involved in the first step of the heptose biosynthesis pathway of LPS. However as yet it is not proven whether *gmhA* is present in all isolates

of *C. jejuni* and *C. coli*, it would seem likely that this gene would be present. For those isolates that did not generate an amplicon, it is possible that gene transfer or mutations have altered the primer binding sites, therefore producing a null result.

This method also proved to be highly reproducible in the isolates that generated the PCR amplicon, as well as being robust and relatively simple and rapid to perform. However not all isolates were able to provide a positive result. A majority of the isolates that had not provided a *flaA* amplicon were also not able to generate the *gmhA* amplicon. This may support the assumption that these isolates are not *C. jejuni* or *C. coli*, but may be another *Campylobacter* spp..

4.4 COMBINATION OF *flaA* AND *gmhA* TYPING.

On its own *flaA* served as a suitable marker for epidemiological studies. Examining the *gmhA* gene from the same sample did not provide the same level of discrimination. However a combination of these two genes results in a higher level of discrimination.

From these experiments a combination of two or more unlinked genes should serve as better epidemiological markers than examining only one gene, as has so often been the case. Comi *et al* (1996) used only one gene, *flaA*, but on the basis of RFLP profiles generated by two different enzymes it was possible to discriminate isolates further. In their study they observed the possibility of developing a technique whereby multiple restriction enzymes (REs) may be used to distinguish *C. jejuni* from *C. coli*. However conservation of gene sequences would be reflected by both REs. By examining two unlinked genes with the same RE it was possible to develop a greater understanding of the true relationship of the isolates. However horizontal gene transfer of one gene may still obscure "true" results.

Amplification of both genes is reproducible and robust. PCR amplification of *flaA* and *gmhA* can often be performed together as the cycling conditions are identical, only the primers differ. Often one isolate was examined for both genes simultaneously, preparing two tubes with all the reagents and their specific primers. This procedure allowed rapid results to be achieved. Multiplexing, the incorporation of all reagents into

one tube, including primers specific for both genes, was not attempted in this study. Amplicons generated by this technique would have been difficult to separate if an isolate generated both a 1.7 kb *flaA* and 1.6 kb *gmhA* amplicon. The combination of two fragments would also have generated too many RFLP fragments to allow accurate classification. Therefore it was chosen to examine these two genes separately.

4.5 LEVEL OF DISCRIMINATION.

The capability of an epidemiological marker to discriminate between isolates is important. In the present study *flaA* was observed to have a D value of 92 % with isolates that provided an amplicon. *gmhA* provided a D value of only 74 %. When these two genetic markers were combined a D value of 92 % was observed. While this is the same as *flaA* alone, further examination found that discrimination of isolates had indeed occurred. Owen *et al* (1994) observed that a combination of *flaA* profiling with either serotyping or biotyping increased discrimination of isolates. These observations were supported in the present study. 17 *flaA* types were further discriminated into 22 types by the addition of *gmhA*.

4.6 MULTILOCUS ENZYME ELECTROPHORESIS (MLEE).

As was observed with combination of the two markers, *flaA* and *gmhA*, upon addition of MLEE, the level of discrimination between isolates increases. Due to the time constraints on this study only a small sample was examined by a second genetic test, MLEE. It was decided to examine the largest *flaA* group, *flaA* 6.

With the analysis of two enzymes used for MLEE it was possible to discriminate these 15 isolates into a further four groups, with 11 isolates appearing identical, therefore suggesting possible relatedness or similarity to each other. However, as has been evident from the previous experiments, the more markers applied to a subset of isolates the more likely the chance of discriminating between them increases. If a further 5-8 enzymes were examined using MLEE it should be possible to divide this group into only a few isolates per cluster, unless the isolates truly are identical. This procedure

however, would be expensive. MLEE proved to have a high level of discrimination, but was a time consuming technique.

4.7 GENE TYPING VS GENOME TYPING.

The flagellar region of *C. jejuni* has been shown to experience recombination and horizontal gene transfer (Wassenaar *et al* 1995, Harrington *et al* 1997). With the demonstration of genetic recombination between flagellin genes, a number of outcomes need to be considered. With recombination an increased immunogenic repertoire will be observed, however this raises questions as to the stability of the flagellin gene as a genetic marker (Harrington *et al* 1997). While recombination can occur within or between flagellin loci, genotypically the organism may remain unaltered. Therefore relationships determined by flagellin gene typing may not accurately reflect true clonal relationships, as would be determined by other genotypic methods (Harrington *et al* 1997).

Horizontal gene transfer may provide an isolate with a "new" *flaA* gene, possibly from another species, therefore making comparisons of the *flaA* gene difficult (Harrington *et al* 1997).

Recombination and horizontal gene transfer may also be possible for the *gmhA* gene. The closely related groups indicate that they have diverged from a common ancestor, while the other types, *gmhA* 3/*gmhA* 7, may have diverged some time ago. Harrington *et al* (1997) observed horizontal gene transfer in isolates of *C. jejuni*. They assumed that recombination had occurred in a one year period, therefore suggesting recent or modern evolution of the gene. The *gmhA* gene may have under gone a modern recombination event. As with *flaA*, it has not been determined whether recombination or horizontal gene transfer has occurred in the isolates examined in this study. Further experiments would need to be carried out to test this hypothesis.

Genome typing appears to be more reliable than gene typing as it measures chromosomal differences. The chromosome is more stable than individual genes, which are subject to recombination. Genotypic results have the potential of providing a more

consistent and reproducible result than biochemical and phenotypic tests. However, genotypic techniques are complex and time consuming, often involving specialised reagents, equipment and staff (Patton *et al* 1991).

Researchers have applied many different typing methods to *Campylobacter* spp.. In New Zealand at the Communicable Disease Center, serotyping, biotyping and macro-restriction fragment length polymorphisms using PFGE were trialled. No one method was found to be ideal (Nicol and Wright 1997). An international standard of typing methods needs to be developed to allow accurate and consistent classification and comparisons of *Campylobacter* spp..

4.8 ENVIRONMENTAL (SHEEP) TESTING.

4.8.1 *flaA* TYPING.

*Dde*I PCR-RFLP analysis of 20 *C. jejuni* isolates from sheep generated four profiles previously observed in the human population. In a study of *flaA* types found in humans, chicken, sheep and bovine, Owen *et al* (1994) observed a common *flaA* type amongst all groups. This supports the observations of the present study that *C. jejuni* strains are present in both the human and sheep populations. Therefore, it was not surprising that a new *flaA* type was not identified amongst the sheep isolates examined in this study. However if a larger sample size was examined it may be possible to identify a *flaA* type that is unique to the animal population.

4.8.2 *gmhA* TYPING.

The 20 isolates were also analysed using *gmhA* PCR-RFLP procedures. Of the 20 isolates 17 (85 %) generated an amplicon, with one classified as *gmhA* 3 (CJS-89). No isolates generated the multiple band profile (*gmhA* 7) under PCR conditions. PCR-RFLP analysis generated three *gmhA* profiles, all previously observed in the clinical isolates of this study. As was observed in the clinical isolates, *gmhA* 1 was the largest *gmhA* class represented in this study.

4.9 CONCLUSIONS.

This thesis identified and characterised strains of *C. jejuni* and *C. coli* that were present in a specific human population using molecular techniques to establish whether isolates were unique to both hospital/non-hospital and environmental populations.

Many researchers have reported the usefulness of *flaA* PCR-RFLP analysis as an epidemiological marker. The present study found this technique to be highly reproducible and discriminative and with the addition of a second gene, *gmhA*, discrimination improved. It was observed that isolates of *C. jejuni* and *C. coli* are not able to be distinguished via *flaA* typing, supporting the observations of Comi *et al* (1996).

The results of this study highlight the advantages of using two genetic markers to identify the isolates of *C. jejuni* and *C. coli* responsible for causing gastrointestinal illness. By understanding what strains are prominent at causing disease it may be possible to target a vaccine at the most common types. The isolates in this study were collected from hospitalised patients which in theory, would be more virulent than strains observed in the community.

This study identified six new *flaA* types that were not observed by Armstrong (1997). It may be possible that these strains are more likely to be present in the hospital environment. Alternatively, these results may indicate a shift in strains present in the environment. Armstrong's study was based in the summers of 1995/96 and 1996/97, therefore direct comparisons are difficult. It would be ideal to compare the results of the present study with isolates from the community during the same period of time. This may determine whether the hospital isolates are different from the community, or if a shift has occurred since Armstrong's study. A survey of strains present in the general community at the same time is currently underway to address these questions.

A secondary aim of this study was to identify the strains present in a hospital environment and generate a database to allow accurate and consistent comparisons to be made to future and past experiments. This study identified common strains of *C. jejuni*

and *C. coli* present in the hospital environment during 1997/98, observing that a dominant type (*flaA* 6) was present in both the hospital and animal environment. *flaA* 14, 16 and 21 were also observed in both environments. Therefore it could be concluded from this study that some strains of *C. jejuni* were restricted only to a human population, however a larger sample size needs to be examined to support these observations.

This study also illustrated the usefulness of *flaA* and *gmhA* as a rapid means for identifying a possible outbreak source. As mentioned previously the three cases from Ashburton may indicate an unreported outbreak.

This study used two different genetic tests that provided reproducible, robust and discriminative results. Levels of discrimination were high, with each subsequent test providing further discrimination to the isolates examined.

4.10 FUTURE EXPERIMENTS.

Future experiments would include 23S rRNA analysis of the isolates that did not amplify under *flaA* or *gmhA* PCR conditions. This would determine whether these isolates were *C. jejuni*, *C. coli* or another thermophilic *Campylobacter* spp.. If they proved to be *C. jejuni* or *C. coli* further examination to see what prohibited amplification using *flaA* specific primers may provide some answers. For example, sequence analysis could be performed to determine if heterogeneity around the primer binding sites exists preventing the primers from annealing and therefore generating an amplicon.

Other experiments could also include the analysis of the remaining isolates with MLEE to determine the distribution in the original sample. More enzymes would be important to examine, as this may provide a further understanding of the distribution of isolates in the clinical environment. The MLEE technique could also be applied to the environmental isolates to determine if they are identical to that observed in the clinical isolates. MLEE analysis of the environmental samples may allow further discrimination to determine whether the strains examined in a hospital population are, in

fact, present in an animal environment. By using MLEE analysis the discrimination of the classification scheme developed in this study could increase further.

REFERENCES

Aarts, H. J. M., L. A. J. T. van Lith, and W. F. Jacobs-Reitsma. (1995). Discrepancy between Penner serotyping and polymerase chain reaction fingerprinting of *Campylobacter* isolated from poultry and other animal sources. Letters in Applied Microbiology. **20:**371-374.

Aeschbacher, M., and J. C. Piffaretti. (1989). Population genetics of human and animal enteric *Campylobacter* strains. Infection and Immunity. **57:**1432-1437.

Aguero-Rosenfeld, M. E., X.-H. Yang, and I. Nachamkin. (1990). Infection of Syrian hamsters with flagella variants of *Campylobacter jejuni*. Infection and Immunity. **58:**2214-2219.

Allos, B. M., and M. J. Blaser. (1995). *Campylobacter jejuni* and the expanding spectrum of related infections. Clinical Infectious Diseases. **20:**1092-1101.

Altekruse, S. F., J. M. Hunt, L. K. Tollefson, and J. M. Madden. (1994). Food and animal sources of human *Campylobacter jejuni* infection. JAVMA. **204:**57-61.

Armstrong, J. L. (1997). Investigation of *Campylobacter jejuni* in Canterbury: Relationships between clinical and freshwater isolates. MSc. Thesis. University of Canterbury, New Zealand.

Aspinall, G. O., A. G. McDonald, T. S. Rajn, H. Pang, S. D. Mills, L. A. Kurjanczyk, and J. L. Penner. (1992). Serological diversity and chamical structures of *Campylobacter jejuni* low-molecular-weight lipopolysaccharides. Journal of Bacteriology. **174:**1324-1332.

Aspinall, G. O., A. G. McDonald, T. S. Raju, H. Pang, L. A. Kurjanczyk, J. L. Penner, and A. P. Moran. (1993a). Chemical structure of the core region of *Campylobacter jejuni* serotype O:2 lipopolysaccharide. European Journal of Biochemistry. **213:**1029-1037.

Aspinall, G. O., A. G. McDonald, H. Pang, L. A. Kurjanczyk, and J. L. Penner. (1993b). Lipopolysaccharide of *Campylobacter coli* serotpye O:30. The Journal of Biological Chemistry. **268:**6263-6268.

Aspinall, G. O., A. G. McDonald, H. Pang, L. A. Kurjanczyk, and J. L. Penner. (1994a). Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: Structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with the Guillain-Barré syndrome. Biochemistry. **33:**241-249.

Aspinall, G. O., S. Fujimoto, A. G. McDonald, H. Pang, L. A. Kurjanczyk, and J. L. Penner. (1994b). Lipoploysaccharides from *Campylobacter jejuni* associated with Guillain-Barré syndrome patients mimic human gangliosides in structure. Infection and Immunity. **62:**2122-2125.

Beer, W., M. Adam, and G. Seltmann. (1986). Monosaccharide composition of lipopolysaccharides from *Campylobacter jejuni* and *Campylobacter coli*. Journal of Basic Microbiology. **26:**201-204.

Bej, A. K., J. L. DiCesare, L. Halt, and R. M. Atlas. (1991). Detection of *Escherichia coli* and *Shigella* species in water by using the polymerase chain reaction and gene probes for *uid*. Applied and Environmental Microbiology. **57:**1013-1017.

Berg, D. E., N. S. Akopyants, and D. Kersulyte. (1994). Fingerprinting microbial genomes using the RAPD or AP-RAPD method. Methods in Molecular and Cellular Biology. **5:**13-24.

Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser. (1988). Experimental *Campylobacter jejuni* infections in humans. The Journal of Infectious Diseases. **157:**472-478.

Blaser, M. J., I. D. Berkowitz, F. M. LaForce, L. B. Reller, and W.-L. L. Wang. (1979). *Campylobacter* enteritis: Clinical and epidemiological features. Annual International Medicine. **91:**179-185.

Blaser, M. J., and L. B. Reller. (1981). *Campylobacter* enteritis. The New England Journal of Medicine. **305:**1444-1452.

Blaser, M. J., D. N. Taylor, and R. A. Feldman. (1983). Epidemiology of *Campylobacter jejuni* infections. Epidemiological Reviews. **5:**157-176.

Brieseman, M. A. (1987). Town water supply as the cause of an outbreak of *Campylobacter* infection. New Zealand Medical Journal. **100:**212-213.

Brieseman, M. A. (1994). The epidemiology of *Campylobacter* in Canterbury, New Zealand. International Food Safety News. **3:**64.

Brooke, J. S., and M. A. Valvano. (1996). Biosynthesis of inner core lipopolysaccharide in enteric bacteria identification and characterization of a conserved phosphoheptose isomerase. The Journal of Biological Chemistry. **271:**3608-3614.

Butzler, J. P., and M. B. Skirrow. (1979). *Campylobacter* enteritis. Clinics in Gastroenterology. **8:**737-765.

Chang, N., and D. E. Taylor. (1990). Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a *Sal*I map of *Campylobacter jejuni* UA580. Journal of Bacteriology. **172:**5211-5217.

Christopher, F. M., G. C. Smith, and C. Vanderzant. (1982). Effect of temperature and pH on the survival of *Campylobacter fetus*. Journal of Food Protection. **45:**253-259.

Comi, G., C. Pipan, G. Botta, L. Cocolin, C. Cantoni, and M. Manzabo. (1996). A combined polymerase chain reaction and restriction endonuclease enzyme assay for discriminating between *Campylobacter coli* and *Campylobacter jejuni*. FEMS Immunology and Medical Microbiology. **16:**45-49.

Corry, J. E. L., D. E. Post, P. Colin, and M. J. Laisney. (1995). Culture media for the isolation of *Campylobacters*. International Journal of Food Microbiology, **26:**43-76.

Cover, C. L., and M. J. Blaser. (1989). The pathology of *Campylobacter* infections in humans. Annual Review of Medicine. **40:**269-285.

Daniell, E. (1994). PCR in the marketplace. *In* K. B. Mullis, F. Ferré, and R. A. Gibbs (eds.), The polymerase chain reaction. Birkhauser, Boston. p. 421-426.

Dickgiesser, N., and D. Czylwik. (1985). Chemically defined media for auxotyping of *Campylobacter jejuni*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A. **260:**57-64.

Doyle, M. P., and D. J. Roman. (1982). Prevalence and survival of *Campylobacter jejuni* in unpasteurized milk. Applied and Environmental Microbiology. **44:**1154-1158.

Eidels, L., and M. J. Osborn. (1971). Lipopolysaccharide and aldoheptose biosynthesis in transkelolase mutants of *Salmonella typhimurium*. Proceedings of the National Acadamy of Sciences, USA. **68:**1673-1677.

Faoagali, J. L. (1984). *Campylobacter* in New Zealand. New Zealand Medical Journal. **97:**560-561.

Fischer, S. H., and I. Nachamkin. (1991). Common and variable domains of the flagellin gene *flaA* in *Campylobacter jejuni*. Molecular Microbiology. **5:**1151-1158.

Fujimoto, S., N. Yuki, T. Hoh, and K. Amako. (1992). Specific serotype of *Campylobacter jejuni* associated with Guillain-Barré syndrome. Journal of Infectious Diseases. **165:**183.

Guerry, P., S. M. Logan, S. Thornton, and T. J. Trust. (1990). Genomic organization and expression of *Campylobacter* flagellin genes. Journal of Bacteriology. **172:**1853-1860.

Hancock, R. E. W., D. N. Karunaratne, and C. Berneggerr-Egli. (1994). Molecular organization and structural role of outer membrane macromolecules. *In* J. M. Ghuysen, and R. Hakenbeck (eds.), Bacterial cell wall. New Comprehensive Biochemistry. Elsevier Science B.V. Chapter 12, 263-275.

Harmon, K. M., G. M. Ransom, and I. V. Wesley. (1997). Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. Molecular and Cellular Probes. **11:**195-200.

Harrington, C. S., F. M. Thomson-Carter, and P. E. Carter. (1997). Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: Implications for the flagellin gene typing scheme. Journal of Clinical Microbiology. **35:**2386-2392.

Hasell, S. K. (1994). Campylobacteriosis: A report for the Ministry of Health. Institute of Environmental Science and Research Limited-Crown Research Institute (CRI).

Hasse, A., M. Schousboe., A. Mee., and A. Hawkins. (1995). Use of molecular typing to identify a nosocomial-acquired *Campylobacter* infection. New Zealand Medical Journal. **108:**112.

Hayashi, K. (1994). Manipulation of DNA by PCR. *In* K. B. Mullis, F. Ferré, and R. A. Gibbs (eds.), The polymerase chain reaction. Birkhauser, Boston. p. 3-13

Herbert, P. D. N., and M. J. Beaton. (1989). Methodologies for allozyme analysis using cellulose acetate electrophoresis: A practical handbook. Helena Laboratories, USA.

Herendez, J., A. Fayos, M. A. Ferrus, and R. J. Owen. (1995). Random amplified polymorphic DNA fingerprinting of *Campylobacter jejuni* and *C. coli* isolated from human faeces, seawater and poultry products. Research in Microbiology. **146:**685-696.

Hill, W. E., S. P. Keaster, M. W. Trucksess, P. Feng, C. A. Keysner, and K. A. Lampel. (1990). Polymerase chain reaction identification of *Vibrio vulnifieus* in artificially contaminated oysters. Applied and Environmental Microbiology. **57:**707-711.

Ho, T. W., B. Mishu, C. Y. Li, G. Y. Gao, D. R. Cornblath, J. W. Griffin, A. K. Asbury, M. J. Blaser, and G. M. McKhann. (1995). Guillain-Barré syndrome in northern China relationship to *Campylobacter jejuni* infection and anti-glycolipid antibodies. Brain. **118:**597-605.

Hughes, R. A. C. (1991). Guillain-Barré syndrome. Springer-Verlag, London.

Humphrey, T. J. (1995). Human *Campylobacter* infections: Epidemiology and control. Science Progress. **78:**135-146.

Hunter, P. R., and M. A. Gaston. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. Journal of Clinical Microbiology. **26:**2465-2466.

Jarosik, C. P., and E. J. Hansen. (1994). Identification of a new locus involved in expression of *Haemophilus influenzae* type B lipopolysaccharide. Infection and Immunity. **62:**4861-4867.

Kaijser, B., and F. Mégraud. (1992). Diagnosis of *Campylobacter* infections. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: Current status and future trends. American Society for Microbiology, Washington DC. p. 89-92.

Karmali, M. A., and P. C. Fleming. (1979). *Campylobacter* enteritis in children. Journal of Pediatrics. **94**:527-533.

Ketley, J. M. (1995). Virulence of *Campylobacter* species: A molecular genetic approach. Journal of Medical Microbiology. **42**:312-327.

Kim, N. W., H. Bingham, R. Khawaja, H. Louie, E. Hani, K. Neote, and V. L. Chan. (1992). Physical map of *Campylobacter jejuni* TGH9011 and localisation of 10 genetic markers by use of pulsed-field gel electrophoresis. Journal of Bacteriology. **174**:3494-3498.

Klena, J. D., S. A. Gray., and M. E. Konkel. (1998). Cloning, sequencing and characterization of the lipopolysaccharide biosynthetic enzyme heptosyltransferase I genes (*waaC*) from *Campylobacter jejuni* and *Campylobacter coli.* Gene (accepted).

Kuroki, S., T. Haruta, M. Yoshioka. (1991). Guillain-Barré syndrome associated with *Campylobacter* infection. Pediatrics and Infectious Disease Journal, **10**:149-151.

Lacey, R. W. (1993). Food-borne bacterial infections. Parasitology. **107**:S75-S93.

Lam, K. M., R. Yamamoto, and A. J. DaMassa. (1995). DNA diversity among isolates of *Campylobacter jejuni* detected by PCR-based RAPD fingerprinting. Veterinary Microbiology. **45**:269-274.

Lampel, K. A., J. A. Jagow, M. Trucksess, and W. E. Hill. (1990). Polymerase chain reaction for detection of invasive *Shigella flexneri* in food. Applied and Environmental Microbiology. **56**:1536-1540.

Linton, D., A. J. Lawson, R. J. Owen, and J. Stanley. (1997). PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. Journal of Clinical Microbiology. **35**:2568-2572.

Lior, H. D., D. L. Woodward, J. A. Edgar, L. T. Laroche, and P. Gill. (1982). Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. Journal of Clinical Microbiology. **15**:761-768.

Lior, H. (1984). New, extended biotyping scheme for *Campylobacter jejuni, C. coli* and "*C. lardis*". Journal of Clinical Microbiology. **20**:636-640.

Martin, W. J. (1994). Infectious diseases. *In* R. B. Mullis, F. Ferré, and R. A. Gibbs (eds.), The polymerase chain reaction. Birkhauser, Boston. p 406-417.

Maslow, J., and M. E. Mulligan. (1996). Epidemiological typing system. Infection Control and Hospital Epidemiology. **17**:595-604.

McMyne, P. M. S., J. L. Penner, R. C. Mathias, W. A. Black, and J. N. Hennessy. (1982). Serotyping of *Campylobacter jejuni* isolated from sporadic cases and outbreaks in British Columbia. Journal of Clinical Microbiology. **16**:281-285.

McSweegan, E., and R. I. Walker. (1986). Identification and characterization of two *Campylobacter jejuni* adhesions for cellular and mucous substrates. Infection and Immunity. **53**:141-148.

Meinersmann, R. J., L. O. Helsel, P. I. Fields, and K. L. Hiett. (1997). Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. Journal of Clinical Microbiology. **35**:2810-2814.

Mennier, J. R., and P. A. D. Grimont. (1993). Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Research in Microbiology. **144**:373-379.

Micheli, M. R., R. Bova, E. Pascale, and E. D'Ambrosio. (1994). Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method. Nucleic Acid Research. **22:**1921-1922.

Mishu, B., A. A. Ilyas, L. Koski, F. Vriesendorp, S. D. Cook, F. A. Mithen, and M. J. Blaser. (1993). Serologic evidence of previous *Campylobacter jejuni* infection in patients with the Guillain-Barré syndrome. Annals of Internal Medicine. **118:**947-953.

Moran, A. P., E. T. Rietschel, T. U. Kosunen, and U. Zahringer. (1991). Chemical characterisation of *Campylobacter jejuni* lipopolysaccharide containing *N*-acetylneuraminic acid and 2,3-diamino-2,3-dideoxy-D-glucose. Journal of Bacteriology. **173:**618-626.

Moran, A. P. (1995). Biological and serological characterization of *Campylobacter jejuni* lipopolysaccharides with deviating core and lipid A structures. FEMS Immunology and Medical Microbiology. **11:**121-130.

Morooka, T., A. Umeka, and K. Amako. (1985). Motility as an intestinal colonization factor for *Campylobacter jejuni*. Journal of General Microbiology. **131:**1973-1980.

Mourean, P., I. Derclaye, D. Gregoire, M. Janssen, and G. R. Cornelis. (1989). *Campylobacter* species identification based on polymorphisms of DNA encoding rRNA. Journal of Clinical Microbiology. **27:**1514-1517.

Nachamkin, I. (1992). *Campylobacter* and *Arcobacter*. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (eds.), Manual of Clinical Microbiology 6th edition. American Society for Microbiology, Washington, DC. p. 483-491.

Nachamkin, I., K. Bohachick, and C. M. Patton. (1993). Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. Journal of Clinical Microbiology. **31:**1531-1536.

Nachamkin, I., H. Ung, and C. M. Patton. (1996). Analysis of HL and O serotypes of *Campylobacter* strains by the flagellin gene typing system. Journal of Clinical Microbiology. **34:**277-281.

Newell, D. G., G. McBride, and J. M. Dolby. (1985). Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *C. jejuni* to human epithelial cell lines. Journal of Hygiene. **95:**217-227.

Nicol, C., and J. Wright. (1997). *Campylobacter* typing in New Zealand. 9th International Workshop on *Campylobacter, Helicobacter* and related organisms. South Africa. p. 13.

Nikaido, H., and M. Vaara. (1985). Molecular basis of bacterial outer membrane permeability. Microbiological Reviews. **49:**1-32.

Nishimura, M., M. Nukina, J. M. Yuan, B. Q. Shen, J. J. Ma, M. Ohta, T. Saida, and T. Uchiyama. (1996). PCR-based restriction fragment length polymorphism (RFLP) analysis and serotyping of *Campylobacter jejuni* isolates from diarrheic patients in China and Japan. FEMS Microbiology Letters. **142:**133-138.

Nuijten, P. J. M., C. Bartels, N. M. C. Bleumink-Pluym, W. Gaastra, and B. A. M. van der Zeijst. (1990a). Size and physical map of the *Campylobacter jejuni* chromosome. Nucleic Acid Research. **18:**6211-6214.

Nuijten, P. J. M., F. J. A. M. VanAsten, W. Gaastra, and B. A. M. van der Zeijst. (1990b). Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. Journal of Biological Chemistry. **265:**17798-17804.

Owen, R. J., J. Hernandez, and F. Bolton. (1990). DNA restriction digest and ribosomal RNA gene patterns of *Campylobacter jejuni*: A comparison with bio-, sero- and bacteriophage- types of United Kingdom outbreak strains. Epidemiology and Infection. **105:**265-275.

Owen, R. J., and J. Hernandez. (1993). Ribotyping and arbitrary-primed PCR fingerprinting of *Campylobacters*. *In* R. G. Kroll, A. Gilmoun, and M. Sussman (eds.), New techniques in food and beverage microbiology. Blackwell Scientific Public., Oxford. p. 265-285.

Owen, R. J., C. Fitzgerald, K. Sutherland, and P. Borman. (1994). Flagellin gene polymorphism analysis of *Campylobacter jejuni* infecting man and other hosts and comparison with biotyping and somatic antigen serotyping. Epidemiology and Infection. **113**:221-234.

Owen, R. J., K. Sutherland, C. Fitzgerald, J. Gibson, P. Borman, and J. Stanley. (1995). Molecular subtyping scheme for serotypes HS 1 and HS 4 of *Campylobacter jejuni*. Journal of Clinical Microbiology. **33**:872-877.

Patton, C. M., I. K. Wachsmth, G. M. Evins, J. A. Kiehlbanch, B. D. Plikaylis, N. Troup, L. Tompkins, and H. Lior. (1991). Evaluation of 10 methods to distinguish epidemic-associated *Campylobacter* strains. Journal of Clinical Microbiology. **29**:680-688.

Patton, C. M., and I. K. Wachsmuth. (1992). Typing systems: Are current methods useful?, *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: Current status and future trends. American Society for Microbiology, Washington DC. p. 110-128.

Pavlovskis, O. R., D. M. Rollins, R. L. Haberberger, A. E. Green, L. Habash, S. Strocko, and R. I. Walker. (1991). Significance of flagella in colonization resistance of rabbits immunized with *Campylobacter* species. Infection and Immunity. **59**:2259-1164.

Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. (1993). Colonization of broiler chickens by waterborne *Campylobacter jejuni*. Applied and Environmental Microbiology. **59**:987-996.

Penner, J. L., and J. N. Hennessey. (1980). Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. Journal of Clinical Microbiology. **12**:732-737.

Perez-Perez, G. I., J. H. Hopkins, and M. J. Blaser. (1985). Antigenic heterogeneity of lipopolysaccharide from *Campylobacter jejuni* and *Campylobacter coli*. Infection and Immunity. **48**:528-533.

Preston, A., D. Maskell, A. Johnson, and E. R. Moxon. (1996). Altered lipopolysaccharide characteristic of the I69 phenotype in *Haemophilus influenzae* results from mutations in a novel gene, *isn*. Journal of Bacteriology. **178**:396-402.

Public Health Report. (1998).

Raetz, C. R. H. (1990). Biochemistry of endotoxins. Annual Review of Biochemistry. **59**:129-170.

Rees, J. H., S. E. Soudain, N. A. Gregson, and R. A. C. Hughes. (1995). *Campylobacter jejuni* infection and Guillain-Barré syndrome. The New England Journal of Medicine. **333**:1374-1379.

Reeves, P. (1994). Biosynthesis and assembly of lipopolysaccharides. *In* J. M. Ghuysen, and R. Hakenbeck (eds.), Bacterial cell wall. Elsevier Science BV. Amsterdam, Netherlands. vol. 27, p. 281-317

Reina, J., M. J. Ros, and A. Serra. (1994). Susceptibilities to 10 antimicrobial agents of 1,220 *Campylobacter* strains isolated from 1987 to 1993 from faeces of paediatric patients. Antimicrobial Agents and Chemotherapy. **38**:2917-2920.

Ribeiro, C. D., M. T. Thomas, D. Kembrey, J. T. Magee, and T. North. (1996). Resistotyping of *Campylobacter*: Fulfilling a need. Epidemiology and Infections. **116**:169-175.

Robinson, D. A. (1981). Infective dose of *Campylobacter jejuni* in milk. British Medical Journal. **282**:1584.

Rollins, D. M., and R. R. Colwell. (1986). Viable but non-culturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Applied and Environmental Microbiology. **52:**531-538.

Ropper, A. H. (1992). The Guillain-Barré syndrome. The New England Journal of Medicine. **326:**1130-1136.

Rosef, O., B. Goudrosen, G. Kapperud, and B. Underdal. (1983). Isolation and characterization of *Campylobacter jejuni* and *Campylobacter coli* from domestic and wild mammals in Norway. Applied and Environmental Microbiology. **46:**855-859.

Ruiz-Palacios, G. M. (1992). Pathogenesis of *Campylobacter* infection: In vitro models. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (eds.), *Campylobacter jejuni*: Current status and future trends. American Society for Microbiology, Washington DC. p. 158-159.

Sahay, P., A. P. West, D. Birkenhead, and P. M. Hawkey. (1995). *Campylobacter jejuni* in the stomach. Journal of Medical Microbiology. **43:**75-77.

Saiki, R. K., T. L. Bugawan, G. T. Horn, K. B. Mullis, and H. A. Erlich. (1986). Analysis of enzymatically amplified b-globin and HLA-DQa DNA with allele-specific oligonucleotide probes. Nature. **324:**163-166.

Salloway, S., L. A. Mermel, M. Seamens, G. O. Aspinall, J. E. Nam Shin, L. A. Kurjanczyk, and J. L. Penner. (1996). Miller-Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD3. Infection and Immunity. **64:**2945-2949.

Sandstrom, E. G., and A. K. Ruden. (1990). Markers of *Neisseria gonorrhoeae* for epidemiological studies. Scandinavian Journal of Infectious Diseases Supplement. **69:**149-156.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systemics. Applied and Environmental Microbiology. **51:**873-884.

Shibata, D. (1994). Preparation of nucleic acids for archival material. *In* K. B. Mullis, F. Ferré, and R. A. Gibbs (eds.), The polymerase chain reaction. Birkhauser, Boston. p. 47-54.

Skirrow, M. B. (1977). *Campylobacter* enteritis: A "new" disease. British Medical Journal. **2:**9-11.

Skirrow, M. B., and J. Benjamin. (1980). Differentiation of enteropathogenic *Campylobacter*. Journal of Microbiological Reviews. **3:**197-718.

Skirrow, M. B. (1990). *Campylobacter*. The Lancet. **336:**921-923.

Stanley, J., N. Baquar, and E. J. Threlfull. (1993). Genotypes and phylogenetic relationships of *Salmonella typhimurium* are defined by molecular fingerprinting of IS*200* and 16S *rrn* loci. Journal of General Microbiology. **139:**1133-1140.

Stanley, J., C. Jones, A. Burnens, and R. J. Owen. (1994). Distinct genotypes of human and canine isolates of *Campylobacter upsaliensis* determined by 16S rRNA gene typing and plasmid profiling. Journal of Clinical Microbiology. **32:**1788-1794.

Stehr-Green, J. K., C. Nicholls, and P. Mitchell. (1991). Waterborne outbreak of *Campylobacter jejuni* in Christchurch: The importance of a combined epidemiologic and microbiologic investigation. New Zealand Medical Journal. **104:**356-358.

Stonnet, V., and J. L. Guesdon. (1993). *Campylobacter jejuni*: Specific oligonucleotides and DNA probes for use in polymerase chain reaction-based diagnosis. FEMS Immunology and Medical Microbiology. **7:**337-344.

Swaminathan, B., and T. J. Barrett. (1995). Amplification methods for epidemiologic investigations of infectious diseases. Journal of Microbiological Methods. **23:**129-139.

Taylor, D. E. (1992a). Genetics of *Campylobacter* and *Helicobacter.* Annual Review in Microbiology. **46:**35-64.

Taylor, D. E., M. Eaton, W. Yan, and N. Chang. (1992b). Genome maps of *Campylobacter jejuni* and *Campylobacter coli.* Journal of Bacteriology. **174:**2332-2337.

Tebbutt, G. M. (1984). A chemotyping scheme for clinical isolates of *Haemophilus influenzae.* Journal of Medical Microbiology. **17:**335-345.

Tenover, F. C., J. S. Knapp, C. Patton, and J. J. Plorde. (1985). Use of auxotyping for epidemiological studies of *Campylobacter jejuni* and *Campylobacter coli* infections. Infection and Immunity. **48:**384-388.

Tenover, F. C., and C. M. Patton. (1987). Naturally occurring auxotrophs of *Campylobacter jejuni* and *Campylobacter coli.* Journal of Clinical Microbiology. **25:**1659-1661.

Tenover, F. C., C. N. Baker, C. L. Fennell, and C. A. Ryan. (1992). Antimicrobial resistance in *Campylobacter* species., p. 66-73. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: Current status and future trends. American Society of Microbiology., Washington DC.

Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. Journal of Clinical Microbiology. **33:**2233-2239.

Thornton, S. A., S. M. Logan, T. J. Trust, and P. Guerry. (1990). Polynucleotide sequence relationships among flagellin genes of *Campylobacter jejuni* and *Campylobacter coli.* Infection and Immunity. **58:**2686-2689.

Totten, P. A., C. M. Patton, and F. C. Tenover. (1976). Prevalence and characterization of hippurate-negative *Campylobacter jejuni* in Kings County, Washington. Journal of Clinical Microbiology. **25:**1747-1752.

Upritchard, H. G. (1997). Identification and classification of the *gmhA* gene in *Campylobacter jejuni.* MSc Thesis. University of Canterbury, New Zealand.

Waegel, A., and I. Nachamkin. (1996). Detection and molecular typing of *Campylobacter jejuni* faecal samples by polymerase chain reaction. Molecular and Cellular Probes. **10:**75-80.

Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios. (1986). Pathophysiology of *Campylobacter* enteritis. Microbiological Reviews. **50:**81-94.

Wallis, M. R. (1994). The pathogenesis of *Campylobacter jejuni.* British Journal of Biomedical Science. **51:**57-64.

Wassenaar, T. M., B. N. Fry, and B. A. M. van der Zeijst. (1995). Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. Microbiology. **141:**95-101.

Welsh, J., and M. McClelland. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acid Research. **18:**7213-7218.

Williams, J. G. K., A. R. Kubelik, K. J. Livaki, J. A. Ratalski, and S. V. Tingey. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research. **18:**6531-6535.

Withington, S. G., and S. T. Chambers. (1997). The cost of campylobacteriosis in New Zealand in 1995. New Zealand Medical Journal. **110**:222-224.

Yuki, N., T. Taki, M. Takahashi, K. Saito, T. Tai, T. Miyatake, and S. Handa. (1994). Penner's serotype 4 of *Campylobacter jejuni* has a lipopolysaccharide that bears a GM1 ganglioside epitope as well as one that bears a GD1a epitope. Infection and Immunity. **62**:2101-2103.

Yuki, N., S. Handa, T. Tai, M. Takahashi, K. Saito, Y. Tsujino, and T. Taki. (1995). Ganglioside-like epitopes of lipopolysaccharide from *Campylobacter jejuni* (PEN 19) in three isolates from patients with Guillain-Barré syndrome. Journal of Neurological Sciences. **130**:112-116.

APPENDIX I

MEDIA

I.i. GENERAL MEDIA.

Unless otherwise stated, all media were sterilised by autoclaving for 20 minutes, 121°C at 120 kPa. Solutions that were labile at this temperature were filter-sterilised through a 0.22 μm filter prior to addition to sterile media.

Campylobacter Blood-Free Selective Agar Base (Modified CCDA-Preston)

	Per litre
2.5 % w/v Oxoid Nutrient Broth No. 2	25 g
0.4 % w/v bacterial charcoal	4 g
0.3 % w/v casein hydrolysate	3 g
0.1 % w/v sodium desoxycholate	1 g
0.025 % w/v ferrous sulphate	0.25 g
0.025 % w/v sodium pyruvate	0.25 g
1.2 % w/v agar	12 g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving. After sterilisation, 1 ml of cefoperazone solution (3.2 mg/l) was added to the medium.

Mueller-Hinton Agar

	Per litre
30 % w/v beef infusion	300 g
1.75 % w/v acid hydrolysate of casein	17.5 g
0.15 % w/v starch	1.5 g
1.7 % w/v Bacto-agar	17 g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving. For culturing of *Campylobacter,* defibrinated sheep blood (5 %) was added after autoclaving.

Brain Heart Infusion Broth

Typical formula (g/L):	Per litre
Beef heart infusion	25 g
Calf brain infusion	20 g
Protease peptone	10 g
NaCl	5 g
Na_2HPO_4:12H_2O	2.5 g
Glucose	2 g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving.

Nutrient Broth No. 2

Typical formula (g/L):	per litre
Beef extract	10 g
Peptone	10 g
NaCl	5 g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving.

Nutrient Broth No. 2 Agar

Typical formula (g/L):	per litre
Beef extract	10 g
Peptone	10 g
NaCl	5 g
Bacteriological agar	15 g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving.

APPENDIX II

BUFFERS AND SOLUTIONS

II.i. COMMON BUFFERS

Solutions requiring sterilisation were either autoclaved for 20 minutes at 121°C at 120 kPa or filter-sterilised through a 0.22 μm filter. All solutions were stored at ambient temperature unless otherwise stated.

50 x TAE	per litre
2.5 M Tris base	242 g
0.11 % w/v Glacial acetic acid	57.1 ml
50 mM Na_2EDTA (pH 8.0)	46.5 g

Dissolved in dH_2O to final volume of 1 litre, pH adjusted to 8.0

1 x TAE

20 ml of 50 x TAE dissolved in ddH_2O to a final volume of 1 litre.

6 x DNA Loading Buffer for Agarose Gel Electrophoresis

50 % v/v Glycerol

0.25 % w/v Bromophenol blue

0.25 % w/v Xylene cyanol FF

Dissolved in dH_2O.

APPENDIX III

MLEE BUFFERS AND ENZYMES

Extraction Buffer (Selander *et al* 1986)

10 mM Tris HCl

1 mM EDTA

0.5 mM NADP

47.15 ml dH_2O

Make up to 50 ml. Adjust pH to 6.8

Agar Overlay (adapted from Hebert and Beaton 1989)

4 g Bacteriological agar

250 ml dH_2O

Heat the mixture until it boils vigorously. This is most easily accomplished by heating for 2-3 min in a microwave on full power. Store covered at 60°C between uses.

Tris Glycine (TG) Buffer (Hebert and Beaton 1989)

30 g Tris base

114 g Glycine

Make up to 1 litre with dH_2O.

Dilute 1:9 [TG:dH_2O] for general use.

Adjust to appropriate pH (e.g., 7.5) with concentrated HCL.

Tris maleate (Catalase Electrode Buffer) (Aeschbacher and Piffaretti 1989)

12.1 g Tris base

11.6 g Maleic acid

3.72 g disodium EDTA

2.03 g $MgCl_2$:$6H_2O$

Make up to 1 liter with dH_2O.

Adjust to pH 7.0 and 7.5 with concentrated NaOH.

Malic substrate (200 ml stock solution)

180 ml dH_2O

20 ml Tris HCl, pH 9.0

3.68 g L-Malic acid

Add ingredients sequentially and mix. Adjust to pH 8.0.

NAD (Stock Solution)

2 mg/ml

Make up to 30 ml with ddH_2O.

NADP (Stock Solution)

2 mg/ml

Make up to 30 ml with ddH_2O.

MTT (Stock Solution)

10 mg/ml

Make up to 10 ml with ddH_2O.

PMS (Stock Solution)

2 mg/ml

Make up to 10 ml with ddH_2O.

$MgCl_2$ (Stock Solution)

20 mg/ml

Make up to 10 ml with ddH_2O.

0.02M Na_2HPO_4

200 ml dH_2O

0.568 g Na_2HPO_4

Add ingredients sequentially, adjust to pH 7.5 with concentrated HCl.

1M $MnCl_2$

100 ml dH_2O

19.79 g $MnCl_2$

Dilute 1:9 for working solution of 100mM.

Peroxidase (Stock Solution)

8.62 mg/ml

Make up to 10 ml with ddH_2O.

O-Dianiside (Stock Solution)

4 mg/ml

Make up to 10 ml with ddH_2O.

L-amino-acid oxidase (Stock Solution)

10 units/ml

Make up to 2 ml with ddH_2O.

Phe-Leu (Stock Solution)

10 mg/ml

Make up to 10 ml with ddH_2O.

Malate Dehydrogenase (MDH) (Hebert and Beaton 1989)

2 ml Tris HCl, pH 8.0*

3 ml NAD (10 % v/v stock)

528 µl Malic substrate

220 µl MTT (2.2 % v/v stock)

220 µl PMS (2.2 % v/v stock)**

2 ml agar

Add all ingredients sequentially into a M^{c}Cartney Bottle and mix gently, add molten agar and mix prior to overlaying cellulose acetate plate.

* Optional ingredient.
** Labile or photosensitive, add immediately before use.

Malate Dehydrogenase $NADP^+$ (ME) (Hebert and Beaton 1989)

1.2 ml Tris HCl, pH 8.0*

3 ml NADP (10 % v/v stock)

528 µl Malic substrate

88 µl $MgCl_2$ (0.9 % v/v stock)

220 µl MTT (2.2 % v/v stock)

220 µl PMS (2.2 % v/v stock)**

2 ml agar

Add all ingredients sequentially into a M^{c}Cartney Bottle and mix gently, add molten agar and mix prior to overlaying cellulose acetate plate.

Peptidase (PEP) (Hebert and Beaton 1989)

2 ml 0.02M Na_2HPO_4 (adjusted to pH 7.5)

176 µl Peroxidase (1.76 % v/v stock)

352 µl o-Dianisidine (diHCl salt) (0.9 % v/v stock)

88 µl $MnCl_2$ (100 mM stock)

352 µl Peptide (use desired dipeptide, e.g., Phe-Leu) (3.52 % v/v stock)

176 µl L-amino-acid oxidase (8.8 % v/v stock)**

2 ml agar

Add all ingredients sequentially into a M^{c}Cartney Bottle and mix gently, add molten agar and mix prior to overlaying cellulose acetate plate.

Catalase (CAT) (Aeschbacher and Piffaretti 1989)

Incubate cellulose acetate plate for 15 min in 50 ml of a solution containing 1.5 ml of a 50 % v/v solution of hydrogen peroxide and 750 µg of sodium sulfite.

Decant solution, rinse cellulose acetate plate with H_2O, and immerse in freshly made 1.5 % v/v solution of potassium iodide.

Mix gently and remove stain solution when white zones appear on dark blue background.

* Optional ingredient.

** Labile or photosensitive, add immediately before use.

APPENDIX IV

S-PLUS

This computer package is used to design dendrograms illustrating the genetic relationship of isolates examined in this study.

>data<-read.table("a:\filename.csv",sep=",",header=T,row.names=NULL)

Reads the data from the 3.5" floppy, containing the Excel file saved in a CSV format.

>data.dist<-dist(t(as.matrix(data[,2:final column])),metric="binary")

[2:final column] tells S-plus the size of the matrix.

>data.clust<-hclust(data.dist,method="average")

Clusters the information created previously.

>win.graph()

Opens the graph interface window. Using the Window menu switch back to the command window.

>plclust(data,clust,hang=-1,labels=names(data[,2:final column]),ylim=c(0,1),main="cluster name")

Produces the cluster in the graphics window. Using the Window menu you can view the cluster. 'main="cluster name"' is what you want to call the cluster.

>win.printer(file="a:\filename.wmf",h=8.27,w=11.69,format="placeable")

Opens a new file to be saved in.

>plclust(data.clust,hang=-1,labels=names(data[,2:final column]),ylim=c(0,1),main="cluster name")

Tells S-plus what to save.

>dev.off()

Physically saves the file to the disk.

APPENDIX V

RAW DATA

Information collected relating to age, sex and geographic location of the patients examined in this study.

(1 to 15 indicate the patients who provided multiple isolates during the course of illness)

DELPHIC NUMBER	AGE	SEX (M/F)	GEOGRAPHIC LOCATION
XT318V	90	F	CHCH
XV898P	35	F	CHCH
XX158F	21	M	CHCH
YG018S 1	21	F	CHCH
YG023Q 1	21	F	CHCH
IG022P 1	21	F	CHCH
XM653F	11	M	CHCH
YJ936T	23	M	CHCH
YO808T 2	64	M	CHCH
YP567F 2	64	M	CHCH
YN438B	78	F	CHCH
YP502F	48	M	CHCH
YU298M	32	F	CHCH
YU304J	10M	M	SELWYN
BB193H	25	F	CHCH
BL821U	15	M	KAIAPOI
BM271M 3	35	M	WOODEND
BN499U 3	35	M	WOODEND
BV374O 4	36	F	CHCH
CM123F 4	36	F	CHCH
CE934Q	35	F	CHCH
CA755I	28	M	CHCH
CI115W	3	F	RAKAIA
CU130P	6M	M	CHCH
ZB375B	36	M	ASHBURTON
ZB632B	51	M	ASHBURTON
DO399V	28	M	OVERSEAS TRAVELLER
DN031T 5	32	M	CHCH
DO332T 5	32	M	CHCH
DO291I 5	32	M	CHCH
EG546O 6	11	M	CHCH
EG761C 6	11	M	CHCH
ET973Q	35	M	OUTPATIENT
EU369T	83	F	CHCH
EX923G	32	F	OUTPATIENT

FD131W	21M	M	OUTPATIENT
ZF320J	33	F	ASHBURTON OUTPATIENT
FZ917J	52	M	CHCH
GF923R	76	F	CHCH
ZG339F	13M	M	ASHBURTON OUTPATIENT
CR828J	30	M	CHCH
HI881O	25	F	CHCH
HW961T	20	M	LINCOLN\LEESTON
ZH604N 7	26	M	ROLLESTON OUTPATIENT
ZH605O 7	26	M	ROLLESTON OUTPATIENT
ZH606P 7	26	M	ROLLESTON OUTPATIENT
ZH641W	25	M	ASHBURTON OUTPATIENT
ZI814K	14	M	ASHBURTON
JF124L	31M	M	CHCH
ZI975L	16	F	ASHBURTON OUTPATIENT
ZJ271C	23	M	ASHBURTON - FROM TAURANGA
ZJ638R	13	M	ASHBURTON OUTPATIENT
LJ155C	22	M	CHCH
LB445E	6	F	CHCH
LG430I	29	F	NTH CANTERBURY
LG794W	17	F	CHCH
LC667G	30	M	CHCH
LB244Q	10	M	CHCH
KX649A 8	29	M	CHCH
KZ386I 8	29	M	CHCH
KV955A	61	F	CHCH
R923	80	M	CHCH
S052	39	F	CHCH
LS897W 9	26	M	KAIAPOI
LS785M 9	26	M	KAIAPOI
LT038S	80	M	CHCH
LT973B	26	M	KAIAPOI
LR181R	7M	M	CHCH
LS466P	22	F	CHCH
MB200L 10	23	M	CHCH
MB321A 10	23	M	CHCH
MB617S	73	M	KAIAPOI
MD672W 11	67	F	RANGIORA
MC715F 11	67	F	RANGIORA
MC591P 11	67	F	RANGIORA
MS179P	?	?	?
PH790A 12	22	F	CHCH
PH844R 12	22	F	CHCH
PH845S 12	22	F	CHCH
PF322Q	64	M	CHCH
PD850L	27	F	CHCH
PA268R 13	75	F	CHCH
MZ347T 13	75	F	CHCH
MM539G	32	F	CHCH

ZN244B	27	F	ASHBURTON
PN728G	23	M	CHCH
ZN474U	28	M	ASHBURTON
QA598G	14	M	CHCH
ZN807W	28	M	ASHBURTON
QZ233U 14	32	M	CHCH
RC703J 14	32	M	CHCH
QP453U	26	M	KAIAPOI
QM664N	20	M	CHCH
RE097T	18	M	CHCH
RF185D	13M	M	CHCH
QZ840M 15	35	M	CHCH
QZ550A 15	35	M	CHCH
RE159O	68	M	CHCH
RC722O	18	F	ASHBURTON
RC317T	26	M	CHCH
ZP028D	76	M	ASHBURTON
QY139W	53	M	CHCH
RC167B	44	F	CHCH
RD177L	49	M	CHCH

Data used to generate the *flaA* dendrogram. 1 indicates band present, 0 indicates band absent. Band 1-23 indicates the number of bands present at different migrations.

Band	flaA 1	flaA 2	flaA 3	flaA 4	flaA 5	flaA 6	flaA 7	flaA 9	flaA 13	flaA 14	flaA 16	flaA 21	flaA 22	flaA 23	flaA 24	flaA 25	flaA 26
1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
6	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
9	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0
12	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0
13	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0
14	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
16	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0
17	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
18	1	1	0	1	0	1	1	0	1	1	1	0	0	1	1	1	0
19	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
21	1	1	0	1	0	0	0	0	1	0	1	0	1	1	1	0	0
22	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
23	0	0	0	0	1	1	1	1	1	0	1	0	0	1	1	1	0

Data used to generate *flaA* and *gmhA* combined dendrogram. 1 indicates presence of a band, 0 indicates absence of a band. 1-17 represents *flaA* type, 18-24 represents *gmhA* type.

Allele	JF124L	IZF320J	EG761C	QM664N	ET972Q	LJ155C	EU369T	KX649A	MB617S	GF923R	BL821U	ZB632B	RC167B	IG022P	ZB375B	YN436B	BM271M	BB193H
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
18(1)	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
19(2)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
20(3)	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0
21(4)	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
22(5)	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
23(6)	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
24(7)	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Data used to generate MLEE dendrogram. 1 indicates *flaA* 6. 2 indicates *gmhA* 1, 3 *gmhA* 5. 4 ME null result. 5 ME allele 1. 6 ME allele 2. 7 MDH null result. 8 MDH allele 1. 9 MDH allele 2. 10 MDH allele 3. 11 MDH allele 4.

Allele	1958	BL821U	CE934Q	CA755I	ZB632B	R923	S052	LT038S	LS466P	MB200L	QP543U	RE097T	RF185D	RC722O
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	0	1	1	1	1	1	1	1	1
3	0	0	0	0	0	1	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	1
5	0	0	0	0	0	1	0	0	0	0	0	0	0	0
6	1	1	1	1	0	0	1	1	1	1	1	1	1	0
7	0	0	0	0	0	0	0	0	0	0	1	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	1
9	0	0	0	0	1	0	0	0	0	0	0	0	0	0
10	1	1	1	1	0	1	1	1	1	1	0	1	1	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	1